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Regulatory molecules at innate and adaptive immune response: PSGL-1 and HDAC6

PhD Thesis

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Regulatory molecules at innate and adaptive immune response: PSGL-1 and HDAC6

This work is presented by graduate in Biology

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CERTIFICA: Que Norman Núñez Andrade, licenciado en Biología por la Universidad Autónoma de Madrid, ha realizado bajo nuestra dirección el trabajo de investigación correspondiente a su Tesis Doctoral con el título: "Regulatory molecules at innate and adaptive immune response: PSGL-1 and HDAC6"

Revisado este trabajo, el que suscribe considera el trabajo realizado satisfactorio y autoriza su presentación para ser juzgado.
Y para que así conste y a los efectos oportunos, firma el presente certificado en Madrid Octubre de 2015.

Fdo. Francisco Sánchez-Madrid

Fdo. Ana Urzainqui Mayayo

Resumen

El sistema inmune comprende dos clases de respuesta: la respuesta de la inmunidad innata y la respuesta de la inmunidad adaptativa. La inmunidad innata es la primera línea de defensa contra muchos microorganismos y además dirige el otro tipo de respuesta; la respuesta de la inmunidad adaptativa, la cual es un tipo de defensa más versátil. En el sistema inmune intestinal, el cual está recibiendo continuamente diferentes antígenos; las poblaciones inmunes innatas y adaptativas desempeñan un papel crucial para mantener la protección contra infecciones a la vez que evitan una respuesta inflamatoria innecesaria. En la primera parte de este trabajo hemos investigado el papel de PSGL1, una glicoproteína que se expresa en leucocitos, en la regulación de la homeostasis y las respuestas inflamatorias en la lámina propria de ratones tratados con DSS para inducir colitis. Hemos observado que en condiciones normales los ratones deficientes para PSGL-1 mostraban unas proporciones alteradas de poblaciones inmunes innatas y adaptativas, así como macrófagos y células dendríticas activadas con presencia de citoquinas proinflamatorias. Se ha descrito previamente que PSGL-1 juega un papel en la inducción de un programa tolerogénico en células dendríticas, y nosotros confirmamos esto mediante la inducción de una expresión menor del complejo mayor de histocompatibilidad clase II (MHC-II) en las células dendríticas y macrófagos cuando un anticuerpo anti-PSGL-1, que actúa como agonista de la P-selectina, es administrado a ratones normales. Además, tras el tratamiento con DSS los ratones deficientes en PSGL-1 desarrollan colitis de forma más temprana y severa que los ratones control, con un incremento de células Th1 y Th17 además de citoquinas pro-inflamatorias. Estos resultados apuntan a PSGL-1 como un regulador del balance entre respuestas tolerogénicas y protectoras.

En la segunda parte de este trabajo hemos evaluado la contribución de HDAC6, una histona deacetilasa citoplasmática con distintas funciones celulares, en el papel de los linfocitos CD8 citotóxicos que contribuyen a la respuesta inmune adaptativa. Se ha demostrado que HDAC6 es necesario para la correcta función citolítica de los linfocitos T CD8, y que este fenotipo se corresponde con un defecto en la exocitosis. Estudios *in vivo* demuestran que los CTLs deficientes para HDAC6 tienen una menor capacidad protectora frente la infección con vaccinia. Cuando se depleciona HDAC6, la señales de activación a través del receptor de células T (RCT) y la producción de IFN-ɣ son similares a las de un linfocito normal. La monitorización de los gránulos líticos en la región proximal, por debajo de la membrana plasmática en la sinapsis inmune, nos ha

revelado una dinámica alterada en el transporte de estos gránulos. Finalmente, hemos demostrado que HDAC6 interacciona con la kinesina-1 cuando se activa el TCR, y esta interacción podría constituir el mecanismo por el cual HDAC6 favorece la eficiencia en el transporte terminal de los gránulos líticos hacia el dominio secretor para completar su exocitosis.

Summary

The immune system is divided in two types of reactions: the innate immune reactions and the adaptive immune reactions. The innate immunity is the first line of defense against many microorganisms; it also drives the subsequent adaptive immune reaction which is a more versatile defense. At the intestinal immune system, which is continuously challenged by different antigens, the innate and adaptive immune populations play a crucial role in maintaining the protection against infection while avoid an unnecessary immune response. In the first part of this work, we investigated the role of PSGL-1, a glycoprotein expressed in leukocytes, in the regulation of homeostasis and inflammatory responses in the lamina propria of DSS-induced colitis mice. We found that, at steady state, PSGL-1 knockout mice showed altered proportions of innate and adaptive immune population, as well as an activated phenotype of macrophages and dendritic cells that mainly produced pro-inflammatory cytokines. Our group has previously described that PSGL-1 plays a role in the induction of a tolerogenic program in dendritic cells. In the present work, we demonstrated that when an anti-PSGL-1 antibody, which acts as an agonist of P-selectin, is administrated to WT mice induces a decrease of class II molecule (MHC-II). Moreover, after DSS treatment, PSGL-1 deficient mice developed colitis earlier and with higher severity than WT mice, with an increment in Th1 and Th17 cells in addition of pro-inflammatory cytokines. These results point to PSGL-1 as a key regulator of the balance between tolerogenic and protective response.

On the second part of this work, we assessed the contribution of HDAC6, a cytoplasmic histone deacetylase involved in many cellular functions, in the cytotoxic function of $CD8^+$ T cells from the adaptive immune response. We demonstrated that HDAC6 is needed for the correct cytolytic function of CD8, and this phenotype corresponds to a defect in the exocytosis. *In vivo* studies demonstrated that $HDAC6^{-/-}$ CTLs have a reduced protective capacity against vaccinia infection. When HDAC6 is deleted, the TCR activation signals and IFN-ɣ production are comparable in HDAC6 deficient and WT mice. Monitoring the lytic granules at the proximal region beneath the plasma membrane at the immune synapse showed altered dynamics in their transport. Finally, we demonstrated that HDAC6 interacts with Kinesin-1 upon TCR activation, and this interaction represent a mechanism through which HDAC6 favors the efficient terminal transport of lytic granules to their secretory domain in order to complete the exocytosis.

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Abbreviations

APC	Antigen presenting cell
cSMAC	Central supramolecular activation cluster
Ck	Citokine
CTLs	Citotoxic T Lymphocytes
cDCs	Conventional dendritic cells
CD	Crohn´s disease
DSS	Dextran sulphate sodium
D.A.I.	Disease activity index
ERM	Ezrin Radixin Moesin
Gzmb	Granzyme B
GALT	Gut associated lymphoid organs
HDAC6	Histone deacetylase 6
IBD	Inflammatory bowel disease
Ig	Inmunoglobulin
ILCs	Innate lymphoid cells
IFN-ɣ	Interferon gamma
IL	Interleukin
IELs	Intraepithelial lymphocytes
KLC1	Kinesin light chain-1
LP	Lamina Propria
MHC	Major histocompatibility complex
MTOC	Microtubule organizing center
NKs	Natural killer cells
NKTs	Natural killer T cells
OT-I	Ovalbumin-specific TCR for MHCI
Prf1	Perforin-1
PMA	Phorbol 12-myristate 13-acetate
pDCs	Plasmacytoid dendritic cells
PSGL-1	P-selectin glycoprotein ligand-1
Tregs	Regulatory T cells
SNARE	Soluble N-ethylmaleimide-sensitive factor accesory protein receptors
TCR	T cell receptor
Th	T helper cells
TGF-β	Transcription growth factor beta
TSA	Trichostatin A
UC	Ulcerative colitis
VACV	Vaccinia virus

INTRODUCTION

1. INTRODUCTION

1.1 PSGL-1: regulatory role at Ulcerative colitis

The gastro-intestinal tract in humans harbors an estimated number of 10^{14} commensal bacteria composed of over 400 species which contribute to the metabolism of nutrients. Intestinal immune cells are located in a unique anatomic environment intimately associated with the epithelium and continually challenged by a myriad of antigens from commensal bacteria, harmful pathogens, nutrients and food antigens that force the immune system to continuously balance tolerogenic and protective immune response (Coombes & Maloy, 2007; Gross et al, 2015).

The host has developed a number of mechanisms to discriminate between these different stimuli, to ensure the protection against infection while avoiding inflammatory response against non-pathogenic antigens (Coombes et al, 2005). Then disruption of this delicate balance results in inflammatory reactions, e.g., hyper-reactivity to food components or inflammatory bowel diseases (IBDs), such as Crohn´s disease (CD) or ulcerative colitis (UC).

1.1.1 Gut structure

Along the intestine, the surface epithelium is continuously renewed by immature cells arising from invaginations known as the crypts of Lieberkuhn, where multipotent stem cells give rise to different types of mature epithelial cells. The vast majority of these cells are absorptive enterocytes but there are also Paneth cells, goblet cells and neuroendocrine cells. The bacteria can be found in the outer but normally do not penetrate the inner layer of mucus. The production of mucus is controlled by IFN-γ, IL-9 and IL-13 (Steenwinckel et al, 2009). This is important because it acts as a physical barrier and has toxic effects for many bacteria. In fact, a defect in the mucus synthesis increases the penetration of commensal bacteria into the epithelial surface of the colon generating enhanced susceptibility to colitis and colon cancer (van der Sluis et al, 2006).

1.1.2 Distribution of immune system in gut

The lamina propria contains B cells, T cells and numerous innate immune cell populations, including DCs, macrophages, eosinophils and mast cells, whereas the epithelium primarily contains T cells. The epithelial layer, in addition of acting as a barrier, is also involved on the sensing of bacteria mediated by the expression of pattern

recognition receptors (Mowat, 2003). Collectively, the intestinal lamina propria and epithelium contain the largest population of T cells, plasma cells and macrophages in the body (Mowat & Agace, 2014). Although they are separated only by a thin basement membrane, the lamina propria and epithelium form very distinct but tightly inter-related immunological compartments (Figure 1).

1.1.2.1 Lymphocyte distribution in the intestine

- Intraepithelial lymphocytes (IELs): They allow the communication with other underlying immune cells; in mice there are 10 to 20 fold higher number of IELs in the small intestine compared to the colon.
- Lamina propria T cells: $CD4^+$ and $CD8^+$ T cells that are found in the lamina propria supposedly derive from naïve T cells activated in secondary lymphoid organs. The $CD4^+$ T cell compartment is diverse, but the distribution of the subsets depends in part on variations in the luminal content. In mice, it has been observed an inverse correlation between the number of Th17 and Treg cells, with a progressively decreasing proportion of Th17 from the duodenum to the colon while Tregs are higher in the colon (Denning et al, 2011). Conversely, in humans, higher proportions of $Th17^+$ cells are found in the lamina propria of the colon. In addition, the frequencies of Th1 and Th2 cells do not vary along the human intestine.
- Intestinal B cells: Lamina propria contains a large number of plasma cells, which density reaches its highest at proximal and distal ends of the tract. IgA producing cells account for nearly 90% of plasma cells in the colon; the other 10% are mostly IgM producing cells (Brandtzaeg & Johansen, 2005).
- Other lymphoid populations in the intestine: Innate lymphoid cells (ILCs) are a recently described population, and they are believed to regulate intestinal immunity, inflammation and GALT development. Invariant T cells are a population that expresses invariant forms of TCR and they include mucosal associated invariant T cells (MAIT cells) and invariant natural killer cells (iNKTs cells). MAIT cells eventually produce cytokines and exert cytotoxic activity, and are selectively activated by infected cells. They constitute a small population throughout the intestine (Le Bourhis et al, 2013). Intestinal iNKTs produce large amounts of IL-4 and IFN-γ, they are activated via TCR

recognition of conserved bacterial glycolipids presented by MHC-I like molecule CD1; they are more numerous in the small intestine than in the colon (Zeissig & Blumberg, 2013).

1.1.2.2 Innate immune cells distribution in the intestine

- Intestinal macrophages: They are the most abundant population of leukocytes in the lamina propria, and are found relatively close to the epithelial surface. They produce large amounts of IL-10, and respond to this cytokine in order to maintain local homeostasis. In fact, this seems to be a property exclusive of intestinal macrophages as, unlike other tissue macrophages, those in the mucosa are derived by continuous replenishment from blood monocytes that are differentiated depending of the local mucosal environment (Bain & Mowat, 2014). It has been suggested that absolute number and relative frequency of macrophages is higher in the colon than the small intestine.
- Intestinal conventional dendritic cells (cDCs): Different subset of dendritic cells have been identified in the mouse lamina propria, with expression of invariant markers like $CD11c^+$, $MHCII^+$ and $F4/80^-$; the combination of CD11b, CD103 and regulatory transcription factors define separated subpopulations. While $CD103^+$ $CD11b^+$ conform the majority of mouse DCs in the small intestine, $CD103^+$ $CD11b^-$ are the major subset in the colon. (Denning et al, 2011; Watchmaker et al, 2014) .
- Plasmacytoid dendritic cells (pDCs): They are present in the intestinal mucosa in a lower number than conventional DCs and are found only in the small intestine in mice and primates. However, pDCs can migrate to the colonic mucosa in response to inflammation. They have been associated with tolerance induction and demonstrated protective role in small intestinal inflammation models (Mizuno et al, 2012).
- Eosinophils: In mice, eosinophil number is higher in the duodenum; it has been reported that eosinophils are important for maintaining the number of IgA-producing plasma cells, DCs and Tregs in the small intestine lamina propria (Chu et al, 2014).
- Mast cells: They are found mostly in the lamina propria and submucosa, and they produce mediators that regulate epithelial barrier integrity, peristaltic

movements, permeability and vascularization. They can also detect microorganisms and, at the colon level, pro-inflammatory mast cells seems to be more abundant (Miller & Pemberton, 2002).

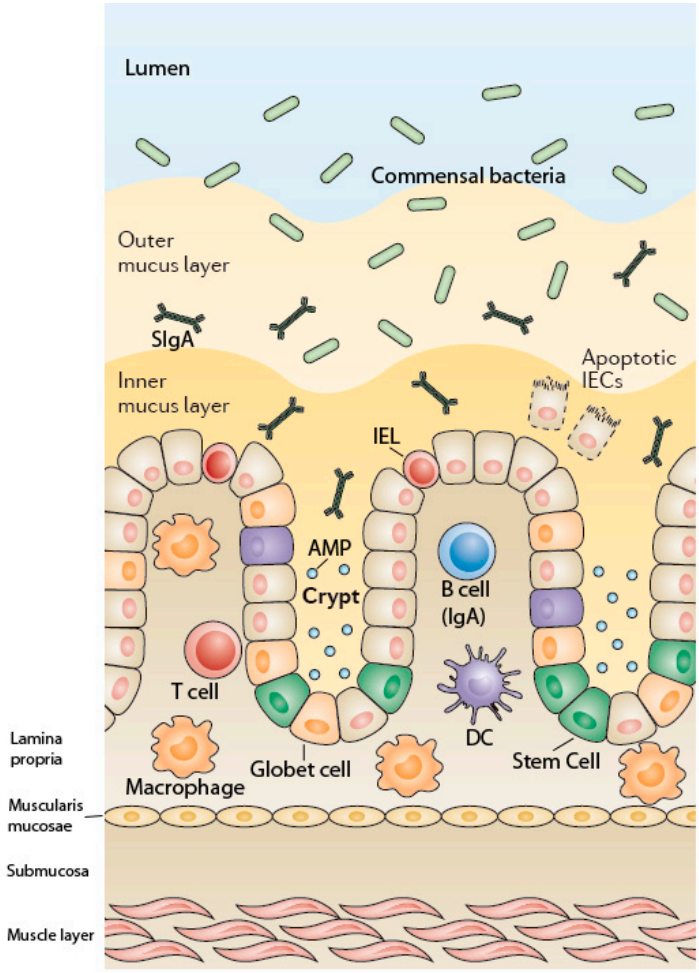

Figure 1. Anatomy of the intestinal mucosa and immune population. The colon has no villi and mucosa consist mainly on crypts with only short regions of flat surface epithelium. Globet cells, which produce a layer of protective mucus, are numerous while Paneth cell are scarce compared to the small intestine. Intestinal epithelial cells (IECs) are produced from stem cells near the bottom of the crypts. Stem cells give rise also to globet cells and Paneth cells, which produce antimicrobial peptides (AMPs). The majority of intestinal immune cells are found at the lamina propria whereas Intraepithelial lymphocytes (IELs) are found lying between epithelial cells. The main function of the surface epithelium is to reabsorb water from faeces and to act as a barrier to commensal microbiota. DC, dendritic cell; SlgA, secretory immunoglobulin A. Modified from (Mowat & Agace, 2014).

1.1.3 Intestinal inflammatory disease

IBDs occur at the distal small (Crohn´s disease) and colon (Ulcerative colitis). They are originated by an aberrant response to commensal bacteria. The incidence of IBDs in western populations is nearly 0.3%. There are numerous animal models for intestinal inflammation; interestingly, they do not develop pathology when the animals are under germ free conditions (Strober et al, 2002). The murine models have been useful to identify the network of principal cytokines with a role in each disease, and are an important tool for research.

Classically, Crohn´s disease has been associated to an inflammatory response driven by Th1 and Th17 T cell population, with increased production of IFN-γ. In contrast, Ulcerative colitis is apparently due to a Th2 response with increased

production of IL-5; however, IL-4 production (Th2 signature) was not increased suggesting that UC is rather a Th2-like disease. IL-12 was initially identified as the master cytokine driving the Th1 response in CD. In fact, patients with CD exhibited increased levels of IL-12 in the lamina propria (Liu et al, 2001). In contrast, TGF-β and IL-6 are the major inducer of Th17 cells that produce IL-17 and IL-22, while IL-23 (produced by macrophages and DCs) interact with differentiated Th17 cells and promote their stabilization and expansion. In fact, it has been reported that differentiation of Th17 cells that do not receive IL-23 lead to IL-10 production (McGeachy et al, 2007). Studies of CD in humans are in agreement with the findings in the murine model, which suggest that Th1 response is quantitatively greater than Th17 (Becker et al, 2006), and beside the fact that both subpopulations may contribute to the inflammatory response, the IL-22 cytokine produced by Th17 cells has been suggested to play a regulatory effect (Zenewicz et al, 2008).

In the same direction, studies of experimental UC demonstrated that IL-13 was increased, but not IL-4 and that this production came from NKT cells rather than from conventional Th2 population (Fuss et al, 2004). In cell transfer colitis as in chemical colitis induced by TNBS or dextran sodium sulfate (DSS), it was seen that IL-17 response was accompanied by an IFN-ɣ response at the earlier onset of the disease. Conversely, oxazolone colitis shows the presence of cells producing IL-4 but not IFN-γ, increased amounts of TGF-β are also detected. Eventually, the inflammation was prolonged but driven by IL-13 secreted by CD4-NKT cells (Fuss & Strober, 2008). Then, it has been accepted that inflammatory response in UC is, greatly in part, generated by NKTs cytotoxicity which are activated by certain glycolipids that are presented by the MHC class I-like CD1; these ligands include self and bacterial lipids. This cytotoxic activity leads to ulceration and inflammation, and this is supported by the fact that IL-13 which causes epithelial cell apoptosis and loss of the barrier function.

1.1.4 Control of intestinal homeostasis

In IBD, the innate immune response plays a critical role. Activated DCs and macrophages secrete several cytokines that eventually trigger differentiation of T cells leading the adaptive immune response. Failed clearance of auto-reactive and over-reactive cells, in addition to imbalance of Treg/Teff cells, are the responsible of the exacerbated inflammation in IBDs. Cytokines IL-1α and IL-β also have up-regulatory and pro-inflammatory activities; they are produced by various cell types as

macrophages and neutrophils, and act synergistically with TNF-α. Also IL-6, a pleiotropic cytokine, contributes with these other cytokines to enhance inflammation. Together, they are implicated in blocking apoptosis of T cells and increasing their proliferation while promote T effector cell differentiation (Figure 2).

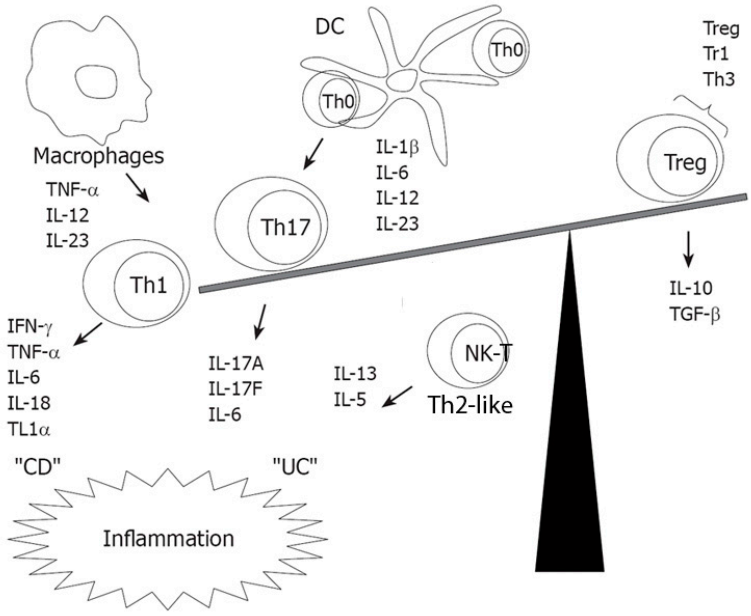

Figure 2. Cytokines imbalance. Pro-inflammatory cytokines from effector T cells vs regulatory cytokines from Tregs. Modified from (Sanchez-Munoz et al, 2008).

DCs are thought to be central to mount either a tolerogenic or an immunogenic response. It has been suggested that conditioning of DC by epithelial cell derived products may be important for the maintaining of intestinal homeostasis. This conditioning would allow DCs to induce tolerogenic responses, IL-10 production, in the absence of infection. Different cell types in intestinal mucosa produce TGF-β; this may regulate negatively DCs function too, by reducing the expression of MHC class II. In fact, there is a constant migration of DCs from the intestinal to the mesenteric lymph node (Msln) and other secondary lymphoid organs. This suggests a constant and active tolerogenic activity performed by intestinal DCs (Huang et al, 2000). Then, the accepted view is that DCs that migrate in the steady-state from the intestine, previously exposed to harmless mucosal antigens, are responsible for the maintenance of tolerance through interaction with T cells in the lymph node. This tolerance may be achieved by deletion or inactivation of autoreactive T cells, the expansion of active Tregs or the acquisition of a regulatory phenotype by naïve T cells.

The $CD4^+CD25^+Foxp3^+$ T cells can be seen in the colonic lamina propria in mice at steady state, which support their constitutive role in the maintenance of tolerance and prevention of aberrant responses to harmless antigens. Indeed, in humans, these Tregs where found at higher density in IBD patients compared to healthy donors (Uhlig et al, 2006). Studies in T cell transfer model of colitis suggested an important role for IL-10 derived from both Treg and non T-cells. The down-regulatory effect of IL-10 includes the inhibition of cytokine production and antigen presentation. Another modulatory cytokine is TGFβ, which is important in the differentiation and maintenance of functional Tregs in periphery.

1.1.5 PSGL1 structure and function

P-selectin Glyprotein Ligand-1 (PSGL1) is a 220-kDa disulfide-linked homodimeric sialomucin, usually located on the tip of microvilli. It is a transmembrane glycoprotein, with an extracellular domain rich in threonine, proline and serine repeats; that bears sites for potential O-glycosylation (Figure 2). The aminoacid sequence of the human and murine transmembrane and cytoplasmic domains is highly conserved. By contrast, the PSGL-1 ectodomain is poorly conserved. PSGL-1 binds the three known selectins; lymphocyte selectin (LSel), endothelial selectin (Esel) and platelet selectin (Psel) (Ley & Kansas, 2004). Different post-translational modifications of PSGL-1 are required for selectin binding, such as sulfation, O- and N- glycosylation, fucosylation, sialylation (Figure 3). In fact, avoiding the catalytic effect of one or more of the involved glycosyltransferases leads to defects in the leukocyte rolling during the extravasation process (Lowe, 2003).

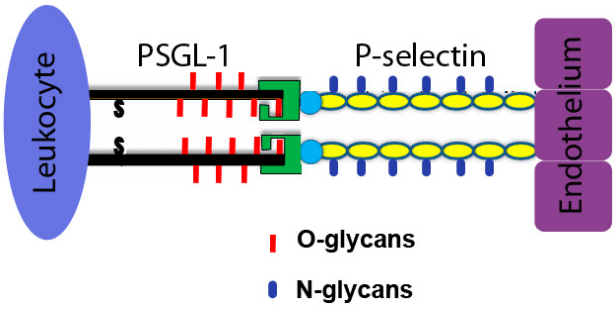

Figure 3. **PSGL-1 schematic structure.** O-glycosylation sites and their modifications condition the binding to PSGL-1 ligands.

At inflammatory situations, Esel and Psel, at endothelium, bind to PSGL-1 initiating the tethering and rolling of leukocytes on the endothelial cells. This represents

the first step of the leukocyte extravasation cascade. These selectins can bind with different affinities to their counter receptors. This sum with the different expression pattern and the signal pathways activated in each case are responsible of the different rolling and attachment characteristics of leukocytes to selectins under physiological conditions (Sreeramkumar et al, 2013). It has been described that both L-selectins and P-selectins bind the same or closely overlapping sites near the amino-terminus of the PSGL-1 ectodomain, however the E-selecting biding site has not been mapped yet (Goetz et al, 1997).

PSGL-1 is constitutively expressed in mature leukocytes. Depletion of PSGL-1 by gene targeting reduces the number of leukocytes interacting with endothelium. Although PSGL-1 binds all the selectins, the *PSGL-1*$^{-/-}$ mice do not present a severe phenotype as the triple selectin knockout *Selp*$^{-/-}$ */Sele*$^{-/-}$ */Sell*$^{-/-}$ mice, confirming that other selectin ligands are important (Robinson et al, 1999).

1.1.6 PSGL-1 signaling

Upon interaction of the PSGL-1 ectodomain with its ligand, the cytoplasmic tail associates and induces phosphorylation of tyrosine kinase Syk through the ERM family proteins in an ITAM-dependent manner. This intracellular signaling drives the activation of the transcription factor *c-Fos* (Urzainqui et al, 2002). Importantly, it has been demonstrated that PSGL-1 plays a role in the induction of peripheral tolerance by activating a tolerogenic program in DCs (Urzainqui et al, 2007b).

DCs play a key role in the induction and regulation of immune response. Upon infection/inflammation, DCs activate naïve T cells but in steady-state DCs remain immature and interact with T lymphocytes inducing anergy and acting as negative regulators. Specifically, engagement of PSGL-1 by P-selectin on immature DCs induces the expression of c-Fos, IDO, IL-10 and TGF-β genes; these DCs exert a tolerogenic effect in naïve T cells inducing differentiation into $CD4^{+}$ $CD25^{+}FOXP3^{+}$ Tregs. In fact, PSGL-$1^{-/-}$ mice showed a lower percentage of natural Tregs in the thymus. Strategies aimed at interfering with T-cell accumulation and/or function in the gut have been employed with success in patients with IBDs (Monteleone & Caprioli, 2010). As Treg populations are key regulators in the homeostasis of intestinal immune system, PSGL-1 may exert a homeostatic role in the gut-associated lymphoid tissue.

1.2 HDAC6: Role at the exocytosis of lytic granules in CD8 T cells

Adaptive immunity involves the differentiation of T cells to effector cells that are able to produce and use different secreted soluble factors and membrane-bound molecules for highly specific intercellular communication. Development of cytotoxic T lymphocytes (CTLs) from activated CD8+ T cells is a key step of the antiviral immune response. CTLs can secrete cytokines but also specifically kill infected cells in the periphery. This targeted cell killing constitutes a form of communication by which cells presenting the appropriate complexes of peptide and major histocompatibility complex are "instructed" to die (Huse et al, 2008)

1.2.1 CD8 cytotoxic response

Cytotoxic cells avoid activation or killing the wrong cell thanks to the specificity conferred to the process by the immunological synapse formed between the CTL and the antigen presenting cell (APC); i.e.: the target cell. The immune synapse functions as a conduit for the transfer of information between the T cell and the APC, and some of this information is mediated by cell surface proteins (Bromley et al, 2001). CTL maturation involves active synthesis and storage of cytotoxic mediators such as perforin and granzymes into the lytic granules, which are derived from lysosomes (Williams & Bevan, 2007).

1.2.2 Cytolytic synapse and MTOC translocation

Cytolytic activity is mediated by the vectorial release of the lytic granule content toward the target cell through the so-called cytolytic synapse, which is organized into a central secretory domain surrounded by a ring of adhesion molecules, analogous to the central supra-molecular cluster (cSMAC) found at the IS between $CD4^+$ T cells and APCs. Within minutes of TCR stimulation, MTOC polarizes just beneath the IS (Geiger et al, 1982). Newly polymerized microtubules radiate with a defined polarity from the MTOC, with "minus" end directed inward and "plus" ends directed outward. Indeed, the MTOC is associated with the Golgi apparatus (Kupfer et al, 1983) and its reorientation aligns the IS with organelles involved in protein secretion.

The cytolytic synapse acts as a focal point for the localized exocytosis of lytic granules. The movement of the lytic granules occurs immediately after TCR stimulation. Granules that are rapidly recruited to the centrosome or microtubule

organizing center (MTOC), are subsequently delivered from the IS-polarized MTOC to an F-actin depleted zone of the plasma membrane within the central secretory domain (Stinchcombe & Griffiths, 2007).

The immune synapse formation involves an initial burst of actin polymerization at the contact site between the T cell and the APC, which then needs to be depleted from F-actin to facilitate the delivery of the lytic granules, and the subsequent activation of integrin-mediated adhesion. This process is dependent on TCR signaling, early signaling effector molecules such as Zap70, Lck and Fyn, adaptors such as LAT (Linker for activation of T cell), SLP-76, and Vav are also required (Ardouin et al, 2003; Kuhne et al, 2003; Martin-Cofreces et al, 2006).

There are other regulators that have been linked to MTOC polarization. Hence, formin-like 1(FMNL1) and mammalian diaphanous 1 (mDia1) are involved in the stabilization of microtubules at the cell membrane (Andres-Delgado et al, 2013). Another issue concerns the functions of the Rho family GTPases. Thus, FMNL1 and Dia1 are directly regulated by Rho and Rac and Cdc42 is also required for MTOC polarization (Faix & Grosse, 2006). The precise concert of all these molecules working together for the polarization response remains unclear.

1.2.3 Polarization of lytic granules

Two main movements are involved in cytolytic granule polarization: initial granule recruitment to the MTOC and MTOC polarization toward the plasma membrane. In this regard, polarization of the MTOC to the CTL contact interface is an essential step for effective cytolytic activity (Stinchcombe et al, 2006). These movements require the activation of the same set of signaling proteins, induced by TCR engagement, but can be uncoupled. The adaptor molecule LAT is recruited to signalosomes and subsequently phospholipase Cγ 1 (PLCγ1) is activated, which cleaves PIP2 and produces diacylglycerol (DAG) and IP3; the latter initiates mobilization of intracellular Ca2+ (Huse et al, 2006; Stinchcombe & Griffiths, 2007). In fact, MTOC polarization requires the tethering of microtubules to remodeled actin cytoskeleton, while granules concentration around the MTOC can occur without cytoskeleton remodeling.

Lytic granules can take either a “short” or “long” path to the cytolytic synapse resulting in distinct patterns of polarization. Both paths use the same intracellular events but with a different spatial and temporal arrangements, and these are regulated by

the kinetics of downstream Ca^{2+} mediated signaling. Rapid signaling causes granule concentration near the MTOC and subsequent delivery by the MTOC translocation directly to the secretory domain (the shortest path). In contrast, a weak signaling leads to late recruitment of granules that move along microtubules to the periphery of the synapse and then relocate tangentially to fuse at the outer edge of the secretory domain (a longer path) (Beal et al, 2009; Poenie et al, 2004). The shortest pathway is associated with faster granule release and more efficient killing than the long pathway (Figure 4). Thus, the kinetics of early signaling regulates the quality of the T cell cytolytic response but the mechanism that control which delivery pathway the lytic granules takes to the synapse remains unknown. It has also been recently described that CTLs may kill their target without centrosomal localization at the IS and that there is a rapid and effective movement of lytic granules that precedes the centrosomal polarization in CTLs (Bertrand et al, 2013). Therefore, part of the control of cytotoxicity may rely on MTOC translocation, but the movement of the granules themselves may constitute the actual mechanism used by CTLs to control their lytic activity.

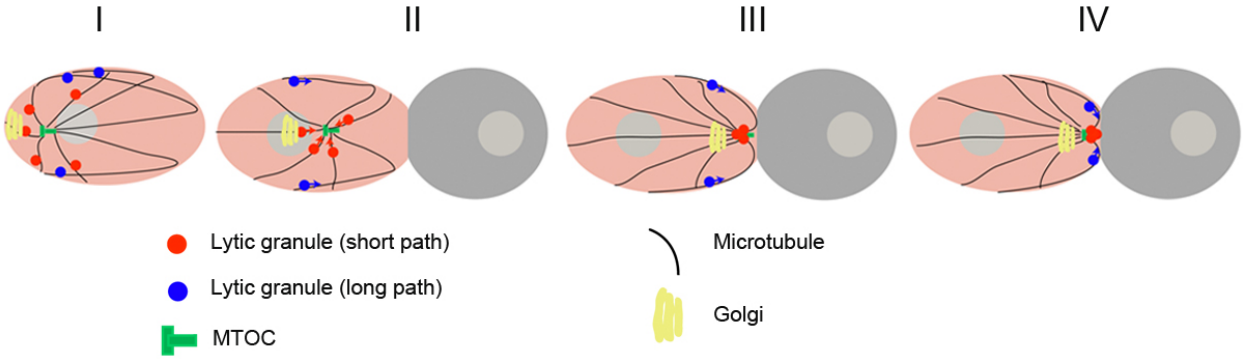

Figure 4. **Polarization of lytic machinery and path to target cell.** In CTLs in the absence of a target cell (I), the MTOC (green) has a perinuclear location, and the lytic granules (red and blue) are distributed all along the microtubules. Upon target cell contact, and TCR recognition, MTOC and Golgi apparatus relocate to the IS. Lytic granules that cluster surrounding MTOC (II) are delivered directly –short path- (III) to the secretory domain where they can fuse with membrane and release their content to target cell (IV). Lytic granules can move along microtubules (II and III) to the periphery of the synapse looping through pSMAC (IV) -long path. Based on (Beal et al, 2009).

The release of lytic granules into the synaptic cleft allows the selective killing by limiting the exposure of surrounding cells to the lytic granule content. CTLs do not exhaust their lytic granules content during the contact with a unique target cell. The lytic granules remain often concentrated around the MTOC even though the latter

moves away from the contact area, which allows the CTLs to rapidly move the lytic granules toward the next target cell (Stinchcombe & Griffiths, 2007).

Trafficking of vesicular compartments is mediated by distinct families of proteins, such as the SNARE proteins (soluble *N*-ethylmaleimide-sensitive factor accessory protein receptors). They conform an important family of helical transmembrane proteins that interact in trans, between members of the family found in either target membranes (t-SNARE proteins) or vesicles (v-SNARE proteins). Upon both kind of proteins form hetero-oligomers through their coiled-coil domain, the vesicle fusion with the membrane is observed and secretion allowed (Jahn et al, 2003; Parlati et al, 2002). SNARE interactions are regulated by different groups of proteins that confer specificity to the trafficking process. These have been extensively studied in neurons; Munc13-1 facilitates the interaction of v-SNARE and t-SNARE and Synaptotagmin regulates the neuronal vesicle fusion that couples calcium influx to the formation of the SNARE complex (Chapman, 2008). Indeed, the Rab family of small GTPases, important for the specification of intracellular traffic, associate with vesicular structures through their carboxyl terminus and recruit various effector molecules and tethering complexes that confer the identity of the vesicle (Zerial & McBride, 2001).

1.2.4 Associated pathologies

Interestingly, some studies have reported genetic disorders that combine immunodeficiency with albinism. Both CTLs and melanocytes secrete lysosome-derived vesicles (lytic granules and melanosomes respectively).

- Griscelli syndrome relates defective CTL function and albinism due a mutation at the Rab27a gene. It is reported that loss of the protein allows the polarization of lytic granules as a response to TCR stimulation, but granules fail to dock at the plasma membrane (Menasche et al, 2000).

- In Hermansky-Pudlak syndrome type 2, the albinism is produced by a mutation in the adaptor protein 3 (AP-3) gene. AP-3 promotes the sorting of lysosome cargo. CTLs deficient for AP-3 have enlarged lytic granules that do not polarize to the immune synapse (Clark et al, 2003).

- Chediak-Higashi syndrome combines albinism and a lymphoproliferative alteration. CTLs show defective exocytosis due to mutation in the gene coding

for LYST (lysosomal trafficking regulator) and they are characterized by enlarged lytic granules (Sepulveda et al, 2015).

1.2.5 Tubulin motors, dynein and kinesin

The tubulin motors mediate the redistribution of lytic granules towards the target cell with exquisite specificity. It has been shown that dynein promotes the transport of lytic granules along microtubules toward the MTOC in a “minus end” direction (Mentlik et al, 2010). This convergence of lytic granules with MTOC occurs long before its polarization toward immune synapse, and is independent of commitment to cytotoxicity. It was demonstrated that lytic granule convergence occurs in cells treated with actin cytoskeleton disrupting drugs such as cytochalasin D or latrunculin A. Given that microtubules are oriented with their plus ends toward the cell membrane, a negatively directed motor anchored to the IS could pull the MTOC toward it, as a mechanism to polarize the ensemble to the IS. The fact that the convergence of lytic granules occurs independently of cytotoxicity, prevents their secretion until gradual MTOC polarization brings them into proximity to the immune synapse.

Eventually, close to the immune synapse, the lytic granules acquire effector exocytic molecules like Rab27a and Munc13-4 that enable the functional maturation of lytic granules (Menager et al, 2007). An additional downstream step that has been demonstrated, Figure 5, includes the recruitment of Slp3 by the granule-associated Rab27a and the interaction with the C-terminal of the kinesin 1 light chain (KLC1). The complex formed by Slp3 and Rab27a and kinesin-1 drives the terminal transport of lytic granules towards the plasma membrane, along the “plus end” direction of the microtubules inserted into the reorganized F-actin network, for directed degranulation at the secretory domain (Kurowska et al, 2012).

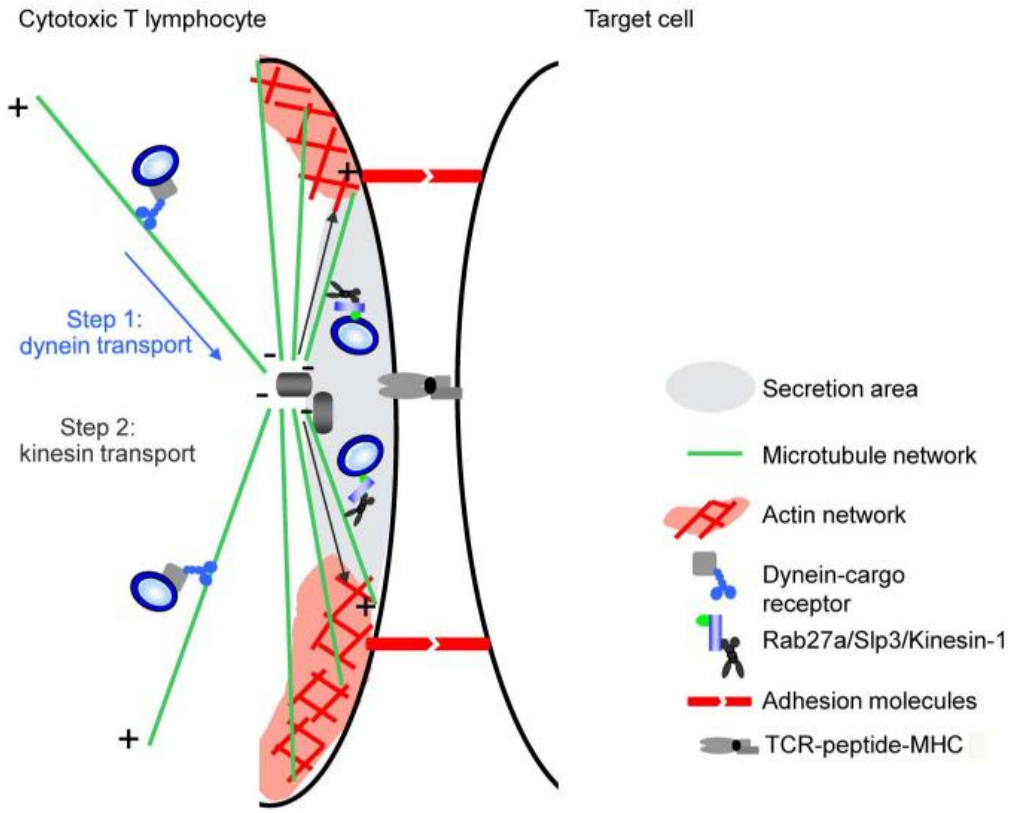

Figure 5. **Terminal transport of lytic granules at IS.** Two successive steps have been described in lytic granule transport at IS formation. A first minus end directed transport dynein-dependent recruits the granules to the MTOC and allows their transport associated with the MTOC translocation bringing them near to the IS. Then a second plus end directed transport kinesin-dependent enables the terminal polarization of cytotoxic granules and their fusion with membrane and release their content. Modified from (Kurowska et al, 2012)

1.2.6 HDAC6 structure

Histone deacetylase 6 (HDAC6) is an ubiquitous cytosolic protein that binds to and deacetylates α-tubulin at lysine 40 *in vivo* and *in vitro* (Hubbert et al, 2002; Valenzuela-Fernandez et al, 2008). The lysine deacetylation is a dynamic and reversible process regulated by the action of antagonistic enzymes, histone acetyltransferases (HATs) and histone deacetylases (HDACs). HDAC6 is a class IIb HDAC that contains two catalytic domains for deacetylation and a C-terminal zinc finger domain (BUZ) that binds free ubiquitin as well as mono- and poly-ubiquitinated proteins with high affinity (Li et al, 2013). Also, HDAC6 harbors: i) a SE motif that confers its cytoplasmic location; ii) a ¨nuclear export signal¨ and a ¨nuclear localization signal¨ that export to cytoplasm or retain in the nucleus the entire or part of the protein, and a iii) dynein binding site between both catalytic domains, Figure 6, (Kawaguchi et al, 2003).

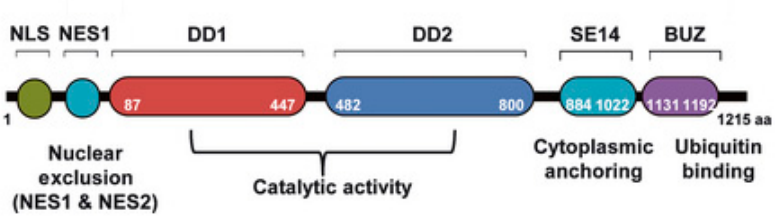

Figure 6. **Functional domains of HDAC6.** HDAC6 contains two catalytic domains (DD1 and DD2), a nuclear export signal (INES) prevents the accumulation in the nucleus and the Ser-Glu tetra-peptide (SE14) which ensures stable anchorage in the cytoplasm. HDAC6 also contains a high affinity ubiquitin-biding zinc finger domain (BUZ). Modified from (Li et al, 2013).

Microtubules are polarized filaments composed of α-tubulin and β-tubulin heterodimers, which are in constant polymerization and catastrophe (depolymerization) events. In this regard, acetylated microtubules have been largely considered as stable microtubules, with long lifetime. HDAC6 also modulates other cytoplasmic substrates, e.g. cortactin, hsp90 or β-catenin.

1.2.7 HDAC6 physiological functions

Through specific deacetylation of its targets, HDAC6 may control different aspects of cell biology, such as:

- *Cell migration*. HDAC6 ablation leads to hyperacetylation of cortactin at its repeat region. This prevents cortactin translocation to the cell periphery, where it may associate with F-actin, impairing the actin-depend cell motility (Zhang et al, 2007).

- *T-regulatory functions*. HDAC6 inhibition and/or depletion promotes suppressive functions of $Foxp3^+$ through the hyperacetylation of HSP90 and disruption of its key chaperone functions. In fact, HDAC6 Tregs express more Foxp3, CTLA-4 and IL10 (de Zoeten et al, 2011).

- *Transcription and signaling*. HDAC6 knockout decreases proliferation, colony formation and invasion in tumor and xenograft models. CD133 links HDAC6 and forms a complex that negatively regulates the endosome trafficking, which allows the deacetylation of β-catenin thereby stabilizing it and preventing its degradation (Mak et al, 2012).

- *$CD4^+$ T cell activation*. HDAC6 mediated α-tubulin deacetylation plays a role in the organization of the immune synapse. In T cells, overexpression of

HDAC6 disorganizes CD3 and LFA-1 at the immune synapse and also impairs MTOC translocation and IL2 production upon activation of T cells. These effects are reverted by TSA treatment (Serrador et al, 2004).

- *Regulation of the aggresome formation*. HDAC6 is selective involved in polyubiquitin-enriched aggresome formation, facilitating the loading of polyubiquitinated misfolded cargo to the dynein motors for their transport to the aggresome (Kawaguchi et al, 2003). Aggresomes are uniquely located close to the MTOC; disruption of microtubule network or dynein motor function prevents the aggresome formation (Johnston et al, 2002). Misfolded proteins are the result of genetic mutations, inappropriate protein assembly, aberrant modifications and environmental stress. These proteins should frequently be poly-ubiquitinated to be degraded at the proteasome; if they aggregate, their degradation by the proteasome fails, promoting cell toxicity (Kopito, 2000).

- *Chemotaxis*. HDAC6 is required for T cell migration. HDAC6 is highly concentrated in regions with protrusive activity in migrating lymphocytes. Its over-expression increases migration and its knockdown diminish their chemotactic capability. Interestingly, the over-expression of a non-functional mutant of HDAC6 resembles the increased migration phenotype observed in cells over-expressing the wild-type form of the protein and its enzymatic inhibition does not decrease the migration as the knockdown does. Therefore, HDAC6 plays a role in the chemotaxis of lymphocytes as a scaffold protein independently of its enzymatic activity (Cabrero et al, 2006).

1.2.8 HDAC6 deletion vs inhibition at the immune response

Mice lacking HDAC6 are viable and fertile and show hyperacetylated tubulin in most tissues; the lymphoid development is normal and seemed to have a moderated impact in the immune response (Gao et al, 2007; Zhang et al, 2008).

Results obtained with the use of inhibitors that show different selectivity for the diverse HDACs, identified to date, do not clarify the precise role of a protein like HDAC6, that can exert different functions beside the enzymatic context, e.g. the scaffold function in the chemotactic capacity of lymphocytes (Cabrero et al, 2006; Valenzuela-Fernandez et al, 2008). In fact, even though multiple efforts in developing specific inhibitors, they have failed in achieving relative good specificities and their use

may lead to uncontrolled, off-target effects due to the number of deacetylase members with different degrees of homology to the desired HDAC (Table 1). By inhibition of deacetylase activity of HDACs it has been reported that some inhibitors abrogate the induction of T cell activation in $CD8^+$ T cells (Mosley et al, 2006; Tsuji et al, 2015). These authors propose that hyperacetylation of HSP90 enhanced its binding to lymphocyte-specific tyrosine kinase (LCK) and disrupted its phosphorylation stage leading to TCR signaling pathway impairment. This suggests that this function may depend on enzymatic activity of HDAC6. However, in $CD4^+$ T cells Trichostatin A (TSA), a pan-inhibitor of HDACs which includes HDAC6, does not abrogate T cell receptor signaling but prevents signaling complexes formed by phosphorylated CD3ζ and ZAP70 from interact to tubulin (Serrador et al, 2004).

Tubacin	**Tubacin is a highly potent and selective, reversible, cell-permeable HDAC6 inhibitor with an IC50 of 4 nM, approximately 350-fold selectivity over HDAC1.**
Tubastatin A	**Tubastatin A is a potent and selective HDAC6 inhibitor with IC50 of 15 nM. It is selective against all the other isozymes (1000-fold) except HDAC8 (57-fold).**
Rocilinostat (ACY1215)	**Rocilinostat (ACY-1215) is a selective HDAC6 inhibitor with IC50 of 5 nM. It is >10-fold more selective for HDAC6 than HDAC1/2/3 (class I HDACs) with slight activity against HDAC8, minimal activity against HDAC4/5/7/9/11, Sirtuin1, and Sirtuin2. Phase 2.**
CAY10603	**CAY10603 is a potent and selective HDAC6 inhibitor with IC50 of 2 pM, >200-fold selectivity over other HDACs.**
Nexturastat A	**Nexturastat A is a potent and selective HDAC6 inhibitor with IC50 of 5 nM, >190-fold selectivity over other HDACs.**
HPOB	**HPOB is a potent, selective HDAC6 inhibitor with IC50 of 56 nM, >30-fold selectivity over other HDACs.**

Table1. List of specific HDAC6 inhibitors

OBJECTIVES

2. OBJECTIVES

2.1 Study of the regulatory role of PSGL-1 in Ulcerative colitis.

2.2 Determination of the role of HDAC6 in the exocytosis of lytic granules in CD8 T cells.

MATERIAL AND METHODS

3. MATERIAL AND METHODS

3.1 Reagents and monoclonal antibodies.

3.1.1 PSGL-1: regulatory role at Ulcerative colitis

The following conjugated antibodies were obtained from BD Pharmingen (San Jose, CA, USA): anti-CD11c-PECy7, F4/80-biotin, I^a-I^e-MHCII -FITC, CD4-PE, CD8-APC, CD25-biotin, B220-FITC, Gr1-APC, DX5α-biotin, CD3-FITC, IL-12-PE, IL-10-PE, IFN-γ-FITC, IL-4-APC, IL-17-PE and streptavidin-PerCP. DSS was from MP Biomedicals, LLC (Illkirch, France). Collagenase IA, dispase, DNAse I and the TRI® Reagent were from Sigma-Aldrich (St. Louis, Mo, USA). The mouse Th1/Th2 10plex Kit was from Bender Medsystems GmbH (Vienna, Austria). Tissue proteins were extracted with T-PER® Tissue Protein Extraction buffer from Thermo Scientific (Rockford, IL, USA), and quantified with the BCA™ Protein Assay Kit (Pierce, Rockford, IL, USA).

3.1.2 HDAC6: Role at the exocytosis of lytic granules in CD8 T cells

The antibodies used in this study were from BD pharmingen (p150-glued, p50-dynamitin), BD (CD4-, CD8-, I^a-I^e-MHCII- and CD44-FITC (fluorescein isothiocyanate); CD11b-, CD11c- CD8- and CD3-PE (phycoerythrin); Gr1-, CD3-, IFN-γ- and CD8-APC (allophycocyanin); biotinylated CD80 and stimulating CD3 (clone 2C11) and CD28 antibodies), Millipore (KLC), Sigma (anti-α-tubulin clone DM1A, vimentin), Cell Signaling (phospho-Erk 1/2, phospho-PLCγ1 (Y783), PLCγ1), Abcam (Cathepsin D) eBioscience (Perforin1 (clone JAW246), lamp1(CD107a)-alexa 647, IFN-γ ELISA), Abnova Bio (Perforin1, rabbit source) and Assay Biotech (HDAC6; reference CO226). Dextramer for H-2KbOVA detection was from Immudex. Reagents from Invitrogen (CMAC (7-amino-4-chloromethylcoumarin), Cell violet and Lysotracker Red probes), Electron Microscopy Sciences (EMS; 16 % paraformaldehyde, electron microscopy grade) were also used. OVA-derived peptides were chemically synthesized (Lifetein) and probed against LPS (lipopolysaccharide ENDOlysa from Hyglos).

3.1 Mice.

3.2.1 PSGL-1: regulatory role at Ulcerative colitis

C57Bl/6 PSGL-1$^{-/-}$ mice were kindly provided by Dr. M.K. Wild and Dr. D. Vestweber (Max Planck Institute for Molecular Biomedicine, Münster, Germany). C57Bl/6 WT mice were obtained from the Jackson Laboratory.

3.1.2 HDAC6: Role at the exocytosis of lytic granules in CD8 T cells

HDAC6 knockout mice were kindly donated by Tso Pang Yao lab´s (Gao et al, 2007) and was intercrossed in a C57BL/6 background to generate wild-type (WT; *hdac6*$^{+/+}$) and knockout (KO; *hdac6*$^{-/-}$) HDAC6 littermates. TCR (Vα2, Vβ5) transgenic mice (OT-I) were crossed to HDAC6 heterozygous mice in order to generate WT and *hdac6*$^{-/-}$ OT-I transgenic mice. *rag1*$^{-/-}$ mice were used for adoptive transfer experiments.

3.3 Methods

3.3.1 PSGL-1: regulatory role at Ulcerative colitis

3.3.1.1 Cell preparation from tissues and analysis

To prepare cell suspensions from LP, colons were extracted, washed with PBS, cut into 1mm pieces and incubated in RPMI medium supplemented with 4% fetal calf serum and 1 mg/ml collagenase IA, 1mg/ml dispase and 40 μg/ml DNAse I in a shaking bath at 37ºC for 1h. Then, cells were washed, suspended in PBS containing 1% BSA,0.5M EDTA, and labeled for cell cytometry analysis. To measure cytokines, colons and mLN were frozen in liquid nitrogen, powdered and suspended in protein extraction lysis buffer. Protein content was quantified with the BCA™ kit and cytokine levels were determined with the cytokine Mouse 10plex Kit, following manufacturer´s instructions.

3.3.1.2 Induction of colitis and disease evaluation.

Colitis was induced by daily administration of 4% or 1% DSS (molecular weight 30,000 to 40,000) dissolved in drinking water. The clinical parameters used to score the disease (Disease Activity Index or DAI) were weight loss, loose stools/diarrhea and presence of occult/gross bleeding (Cooper et al, 1993). Animals were weighed before starting the treatment (initial weight) and every day until the end of the treatment. Animals were also checked daily for stool consistency and for the presence of blood in

the stools. At the end of treatment, colons were processed for histological analysis, to check for the presence of infiltrate in the LP, or disaggregated and processed to study the immune cells present in the colonic LP infiltrate.

3.3.2 HDAC6: Role at the exocytosis of lytic granules in CD8 T cells

3.3.2.1 Quantitative real-time PCR. Total RNA was isolated using an RNeasy kit (Qiagen). RNA samples were reverse-transcribed using random primers and SuperScript II reverse transcriptase and qPCR was done by nonspecific product detection (SYBR Green; Applied Biosystems) of cDNA. Primer sequences are: GzmB, forward, 5′-TCACAAGGACCAGCTCTGTCCT-3′, reverse, 5′-GTTGGGTTGTCACAGCATGG-3′; Eomes, forward, 5′-CCCCTATGGCTCAAATTCC-3´, reverse, 5´-CCAGAACCACTTCCACGAA-3´; Perforin, forward, 5′- GAGAAGACCTATCAGGACCA -3′, reverse, 5′-AGCCTGTGGTAAGCATG -3′; Cathepsin D, forward, 5′-CTGAGTGGCTTCATGGGAAT -3′, reverse, 5′- CCTGACAGTGGAGAAGGAGC -3′.

3.3.2.2 In vitro degranulation assay. Cell degranulation was measured by monitoring the expression of the lysosomal marker CD107a, EL4 pulsed with 1μM of SIINFEKL were used to stimulate $CD8^+$ OTI cells in the presence of Monensin 5mM and anti-CD107a Alexa 647 antibody during 3h at 37 ºC in 5%CO_2. Thereafter, cells were stained with anti-CD8 PE and anti-CD44 FITC (BD Biosciences), and analyzed by flow cytometry. Data were analyzed with FlowJo Version 7.6.5 software (TreeStar).

3.3.2.3 Cell conjugate formation and confocal analysis. EL-4 cell line was loaded with the CMAC cell tracker (10 μM) and pulsed with the specific OVA agonist peptide SIINFEKL for 2 h at 37 ºC. Target cells (1×10^6 cells) were mixed with the $CD8^+$ CTLs (1:1) and plated onto Poly-L-Lys-coated slides (50 μg/ml). Cells were allowed to settle for the times indicated at 37 °C, fixed with 4% paraformaldehyde in PHEM (60 mM PIPES, 25 mM Hepes, 5 mM EGTA, 2 mM $MgCl_2$), and permeabilized for 2 min at room temperature with 0.2% Triton X-100 in immunofluorescence solution (PHEM containing 3% BSA, 100 μg/ml γ-globulin and 0.2% azide) when needed. Preparations were blocked for 30 min with immunofluorescence solution and stained with the indicated primary and Alexa Fluor 488, 568 or 647-labeled secondary

antibodies, except for the fluorochrome-conjugated primary antibodies (FITC-anti-α-tubulin, 647-CD3ε, 647-lamp1(CD107a). Cells were mounted on Prolong Gold (Invitrogen) and analyzed under a Leica SP5 confocal microscope (Leica) mounted on an inverted DMI6000 microscope fitted with a HCX PL APO 63x/1.40-0.6 oil objective. Co-localization analysis was performed with Imaris Software; the fluorescence corresponding to the CTL was analyzed, excluding volumes corresponding to the target cell. Briefly, an image mask was generated from the CMAC channel (target cell) and substracted from the Prf1 and CD107a (Lamp1) channels prior to the calculation of co-localization volumes and the Mander's coefficient (absolute intensity of fluorescence values).

3.3.2.4 Total internal reflection fluorescence (TIRF) video microscopy. $CD8^+$ CTLs were allowed to settle onto glass bottom dishes (No 1.5 Mattek; Ashland, MA, US) coated with anti-CD3 (10 µg/ml) and anti-CD28 (3 µg/ml). Recording was initiated upon cell adhesion to the plate, and cells were visualized with a Leica AM TIRF MC M system mounted on a Leica DMI 6000B microscope coupled to an Andor-DU8285_VP-4094 camera fitted with a HCX PL APO 100.0x1.46 OIL objective. Images were processed with the accompanying confocal software (LCS; Leica). The laser penetrance used was 90 nm (561 nm laser). Time-lapse settings were optimized for each type of experiment and are specified throughout the text. Synchronization was performed with the accompanying Leica software, while Imaris® software and Image J were used respectively for 3D analysis and preprocessing. The study of the mechanical properties of the lytic granules was performed instead with a user-customized routine developed in Python, using the necessary packages for image analysis and numerical calculations (and for the graphical interface). To detect lytic granules we developed a blob-like structure detection algorithm working on the intensity image and on its first derivatives; detection parameters were kept constant for all the analyzed stacks. Granules detected in each frame were tracked along the stack looking at the distances of their centers of mass: manual adjustments were allowed too. After tracking, for each of the identified granules, the software records their average fluorescent intensity and their surface for each time step. Diffusion coefficient for each granule was estimated as the ratio between the surface of the smallest polygon including the whole trajectory and the number of time steps of the trajectory itself. The software maybe freely downloaded from: https://dl.dropboxusercontent.com/u/4050954/VesiclesAnalyser.zip

3.3.2.5 Vaccinia virus (VACV) infection and virus titration. Mice were skin scarified with the virus ($2x10^6$ PFU/mouse), the tail skin area was gently scratched 25 times with a 28 1/2 G needle. For virus titration, the scarified tails were mechanical disaggregated in 1ml of PBS. After 1 freeze-thaw cycles and sonication, serial dilutions of the tail homogenates were added to monolayers of CV-1 cells seeded in 24 well plates. Cristal violet was used to stain the cells 24 hours later. We observed a detection limit of 5 PFU/tail, the number of plaques was multiplied by the reciprocal of sample dilution and converted to p.f.u./g.

3.3.2.6 In vitro cytotoxicity assay. EL4 targets were incubated with 1 μM Cell Violet and pulsed with 1 μM SIINFEKL or with 0.1 μM Cell Violet and no SIINFEKL, washed, and used as targets. These targets were mixed with different dilutions of effectors, as indicated, and plated by triplicate in a 96-well U-bottom plate. Following incubation for 4 hours at 37°C, Propidium iodide (or PI) was added to exclude dead cells and samples were analyzed by flow cytometry. The mean percentage of survival in antigen-loaded targets was calculated relative to antigen-negative internal controls in each sample. The percentage of specific lysis was calculated using the following equation: percentage specific lysis = 100 * (1 - (% Cell Violet 1μM/ % Cell Violet 0,1μM). All data were adjusted to the basal specific lysis in absence of effector cells.

3.3.2.7 In vivo cytotoxicity assay. WT and *hdac6*$^{-/-}$ mice were immunized by i.p. injection of bone marrow dendritic cells pulsed with 1μM of SIINFEKL and LPS 1μg/ml for 1h. After 7 days, CD45.1 splenocytes were used as targets and split into two populations labeled with 1 μM Cell Violet and pulsed with 1 μM SIINFEKL or with 0.1 μM Cell Violet and no SIINFEKL, washed, and used as targets, and injected i.p. into recipients. Cells were recovered 16 hours later by peritoneal lavage and *in vivo* killing measured by flow cytometry.

RESULTS

AND

DISCUSSION

4. RESULTS AND DISCUSSION

4.1 PSGL-1: regulatory role at Ulcerative colitis

4.1.1 Distribution and activation state of LP leukocytes

Commensal bacteria (microbiota) normally exist in a symbiotic relationship with the host intestine, but members of the microbiota can opportunistically invade mucosal tissues, causing serious problems in immunodeficient individuals. To ensure protection of the host against such opportunistic bacterial invasions, the balance between tolerance and immunity must be tightly regulated, and this intestinal homeostasis is maintained by the immune system (Hooper & Macpherson, 2010; Tlaskalova-Hogenova et al, 2004).

We analyzed the phenotype of the leukocytes in the colonic LP of PSGL-1 deficient mice and found a relative increased numbers of granulocytes, CD8+ cells and B cells (Figure 1). In contrast, a diminished number of macrophages ($F4/80^+$), DCs (lineage negative, $CD11c^+$) and NK cells ($DX5\alpha^+$) was observed in the LP of the KO mice.

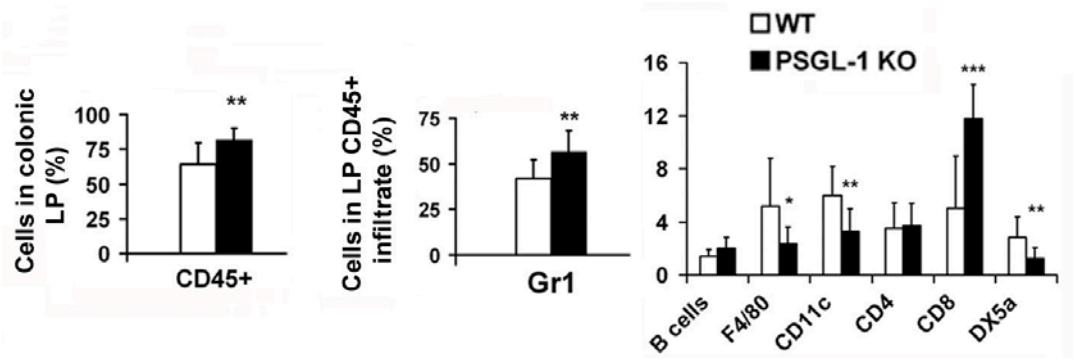

Figure 1. **Resident leukocyte subpopulations in the colonic lamina propria (LP) of PSGL-1$^{-/-}$ mice.** Cell suspensions were obtained from colons of WT and PSGL-1 KO mice, stained with a cocktail of antibodies and analyzed by FACS. Percentage of CD45+ cells in the LP infiltrate and percentages of granulocytes (Gr1+), B cells (B220+), macrophages (F4/80+), DC (CD11c+), CD4+ T cells, CD8+ T cells and NK cells (DX5+) present in the CD45+ cell population. Data are means ± SD (n=10 mice).

It is very likely that the lower proportion of dendritic cells ($CD11c^+$), macrophages ($F4/80^+$) and NKs ($DX5\alpha^+$) at the steady state condition is caused by a reduced recruitment of these cells to the LP. By contrast, the percentage of total leukocytes ($CD45^+$) including granulocytes ($Gr1^+$) and $CD8^+$ T cells showed relative higher proportions. This phenotype suggests two possibilities: that it may occur an intrinsic increased proliferation of the resident sub-population in the LP, or that some

extravasation signal may overcome the defect in their recruitment. In these populations PSGL-1 seems to be not crucial for their homing to LP.

In addition, we could not detect DCs from lamina propria of PSGL-1 knockout mice expressing low levels of MHC-II (data not show). Moreover, all PSGL-$1^{-/-}$ macrophages expressed the co-stimulatory molecule CD86, whereas only 20% of WT macrophages were positive for this molecule (Figure 2).

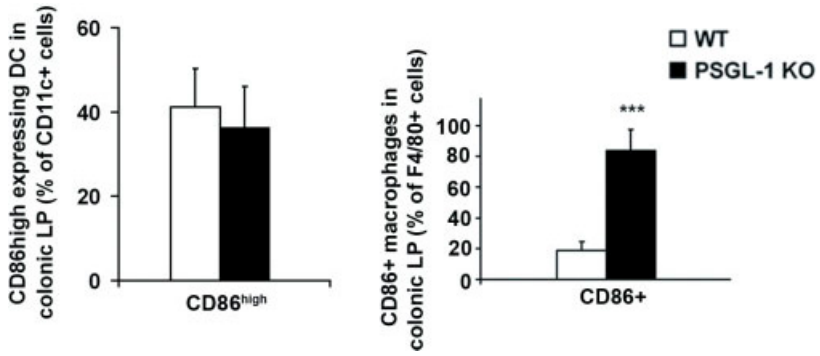

Figure 2. **Expression of activation markers in myeloid cells from LP**. The bar chart shows the percentage of F4/80+ cells that express the co-stimulatory molecule CD86 (mean ± SD; n = 5 mice).

The higher rate of activated macrophages ($CD86^{+}$) observed in lamina propria suggests the presence of an activation signal even at steady state. Given that these knockout mice do not develop spontaneous disease, the contribution of this activated population seems to be necessary or at least it is not impairing the homeostasis in gut. These data suggest that in the colonic LP of PSGL-$1^{-/-}$ mice most DCs and macrophages are in activated state, likely acting as efficient antigen presenting cells. Macrophages are inferior to DCs in their ability to prime naïve T cells (Mildner & Jung, 2014); this characteristic may be due to their ability to rapidly degrade ingested proteins, which impairs their capacity to retain antigens for presentation.

4.1.2 Effect of anti-PSGL-1 antibody on the recruitment of leukocytes to the LP

In order to characterize the leukocyte subsets that require PSGL-1 for their recruitment to the colonic LP, we treated WT mice *in vivo* with the 4RA-10 anti PSGL-1 antibody. The *in vivo* blockade of PSGL-1 in the lamina propria at steady state was analyzed 18 hours after the administration of the last dose of the antibody. The binding of the 4RA-10 antibody resembles the functional silencing of PSGL-1, when this is deleted, blocking interaction with the natural ligands. Anti-PSGL-1 treatment reduced

the total number of macrophages, DCs and B cells in the colonic LP which is consistent with proportions observed in PSGL1$^{-/-}$ mice, whereas other leukocyte subsets remained unchanged (Figure 3A).

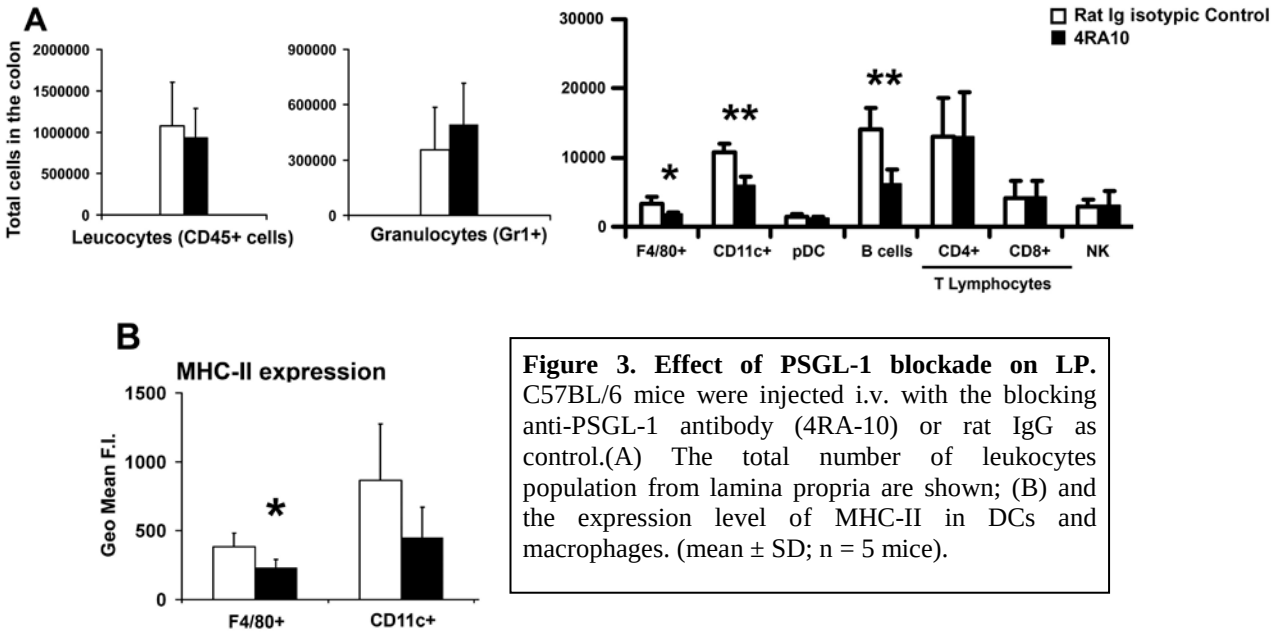

Figure 3. Effect of PSGL-1 blockade on LP. C57BL/6 mice were injected i.v. with the blocking anti-PSGL-1 antibody (4RA-10) or rat IgG as control.(A) The total number of leukocytes population from lamina propria are shown; (B) and the expression level of MHC-II in DCs and macrophages. (mean ± SD; n = 5 mice).

These data are in accordance with our previous results and other reports demonstrating the importance of PSGL-1 in the recruitment of lymphocytes to LP (Haddad et al, 2003). In fact, it has been reported the use of immune-blocking of PSGL-1 antibody to attenuate established murine colitis by reduction of leukocytes recruitment (Rijcken et al, 2004). Therefore, since the balance between some subpopulations is broken while other maintain their proportions when deleting or blocking PSGL-1, suggest that there are some specific populations which are more prone to impair their homing when PSGL-1 interaction with its counter ligands does not occur at steady state conditions. In our experiments we found that specifically DCs, macrophages and B cells impair their homing when PSGL-1 is blocked.

The treatment with anti-PSGL-1 also resulted in a diminished expression of MHC-II by macrophages and DCs (Figure 3B), indicating that 4RA10 acts as an agonist of P-selectin and generates an equivalent signal of the interaction with the selectin-ligand, concurring with our previous results showing that signals through PSGL-1 result in induction of a tolerogenic phenotype with low class II expression in DCs. Our results suggest that PSGL-1 could be playing a dual role in the recruitment of leukocytes but also contributing to the activation state of DCs and macrophages in the lamina propria.

4.1.3 Study of environment in colonic LP, balance of Th1, Th2, Th17 and Tregs.

We then assessed the profile of cytokine production by resident DCs and macrophages in the colonic LP of WT and *PSGL-1*$^{-/-}$ mice under steady state conditions. As shown in Figure 4, most DCs and macrophages from WT mice synthesized IL-10, with only a small percentage of cells producing IL-12, IL-4, IL-17 or IFN-γ. In contrast, in PSGL-1 deficient mice, the number of IL-10+ DC and macrophage was markedly reduced, whereas the proportion of cells producing IL-12, IL-4 and IL-17 was increased, a cytokine profile that may favor the generation of inflammatory phenomena.

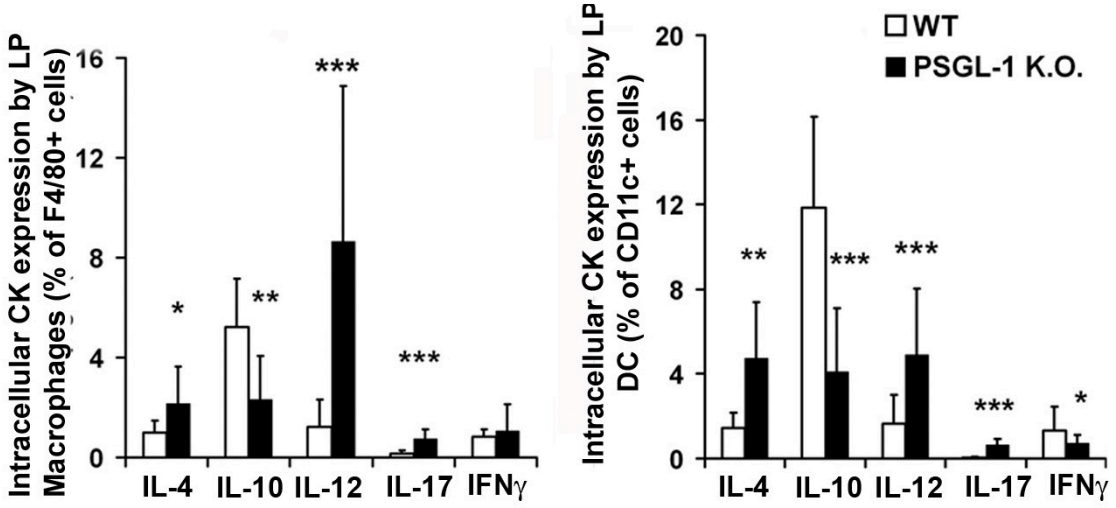

Figure 4. **Innate cells populations in *PSGL-1*$^{-/-}$ mice at steady state.** Percentage of different macrophage (F4/80+) and DC (CD11c+) effector subpopulations resident in the CD45+ infiltrate of colonic LP from WT and PSGL-1 KO mice. Cells were analyzed by FACS after intracellular staining for IL-4, IL-10, IL-12, IL-17 or IFN-γ. Data are means ± SD (n=6 mice).

Dendritic cells and macrophages have distinct, yet complementary roles in maintaining gut homeostasis and immune defense. Dendritic cells, with their migratory capacity, translocate from the lamina propria to the gut-draining mesenteric lymph nodes and Peyer´s patches where they present antigens to B cells and naïve T cells and polarize them toward effector fates. Macrophages are believed to contribute to the local clearance of bacteria, translate alert signals to other immune cells, secrete cytokines and re-stimulate T cells to maintain the homeostasis in the lamina propria.

The cytokine profile and phenotype of *PSGL-1*$^{-/-}$ LP macrophages and DC indicate that they are in an activated state (CD86 and MHC-II expression and enhanced synthesis of IL-4, IL-12 and IL-17, with a diminished production of IL-10), a

phenomenon that could be causally associated with the increased numbers of granulocytes, B cells and CD8+ T cells.

It has been widely described that IL-10 is a potent suppressor of classical macrophage activation (Bogdan et al, 1991), and animals deficient in this cytokine show spontaneous intestinal inflammation (Kuhn et al, 1993) as well as enhanced synthesis of the pro-inflammatory cytokines IL-12 and TNFα (Gazzinelli et al, 1996). Consistently, in the LP of *PSGL-1*$^{-/-}$ mice, the presence of IL-10 producing DCs and macrophages is markedly reduced.

Several studies in IBDs models have shown that most immunoregulatory events are controlled by T-cells, and the activation is antigen-dependent, originated from intestinal bacteria (Ergin et al, 2008). For this reason, we also studied the characteristics of resident T lymphocytes in the colonic LP. As shown in Figure 4B, an enhanced number of IFN-γ+ or IL-4+ CD4 T lymphocytes was detected in *PSGL-1*$^{-/-}$ mice. In addition, an increased proportion of IL-4^{+} CD8^{+} T cells were observed in these animals (Figure 5).

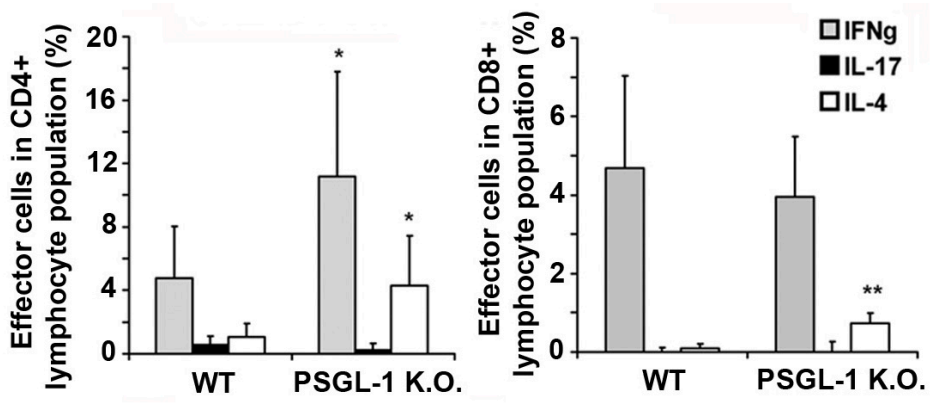

Figure 5. **T cell population in *PSGL-1*$^{-/-}$ mice at steady state.** Percentage of CD4^{+} and CD8^{+} effector subpopulations in the CD45^{+} infiltrate of colonic LP from WT and PSGL-1 KO mice. Cells were analyzed by FACS after intracellular staining for IFN-ɣ (Th1), IL-17 (Th17) and IL-4 (Th2). Data are means ± SD (n=6).

T cells may accumulate in the lamina propria as a result of multiple mechanisms, including enhanced recruitment of cells from the bloodstream, sustained cell cycling and diminished susceptibility of cells to undergo apoptosis. Regarding regulatory cells, the percentage of T cells with a regulatory phenotype (CD25^{high} Foxp-3^{+}) was significantly reduced in the LP of PSGL-1 deficient mice (Figure 6).

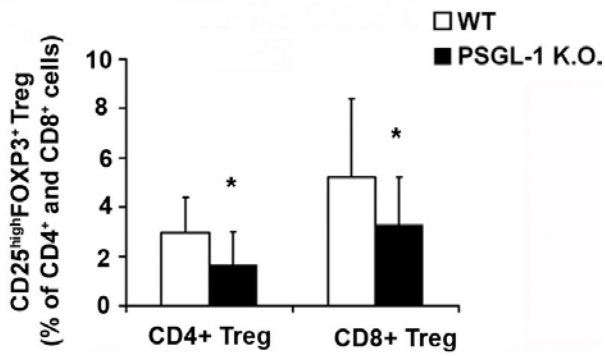

Figure 6. Treg subpopulations in the lamina propria. Percentage of Tregs (CD25high Foxp-3$^+$) in the CD4$^+$ and CD8$^+$ subpopulations of colonic LP CD45$^+$ infiltrate. Data are means ± SD (n = 12 mice).

The local cellular, microbiological and cytokine milieu affect the immunophenotype and function of Tregs. Indeed, the homeostasis and control of colitis depends on the presence of the immunosupresive cytokines IL-10 and TGF-β, both produced by Tregs. IBDs models are accompanied by the accumulation of dendritic cells in the mesenteric lymph nodes as well as locally in the colon. As it was previously reported, the stimulation of PSGL-1 on the DCs with P-selectin enhance the generation of tolerogenic T cells. Then, it is probably that reduction of CD25^{high} Foxp-3$^+$ T cell is due to the disrupted tolerogenic program of the DCs when PSGL-1 signaling is absent.

It is also feasible that the relative low proportion of Tregs observed in PSGL-1 KO mice may be enough to limit the proliferation of Th1, Th2 and Th17 effector cells under steady state conditions (Asseman et al, 1999; Asseman et al, 2003), preventing an spontaneous colitis. In this regard, it is also possible that the impaired leukocyte recruitment in *PSGL-1*$^{-/-}$ mice may prevent the immune surveillance and therefore *PSGL-1* would contribute to control the response to commensal microbiota.

4.1.4 Experimental colitis induced by DSS in PSGL-1 deficient mice

To assess the influence of the altered gut homeostasis of PSGL-1 deficient mice on the initiation and progression of inflammatory bowel disease, we used the dextran sulfate sodium salt (DSS) induced colitis model. Addition of 4% DSS to drinking water causes acute disease with severe clinical and histological symptoms (Cooper et al, 1993). Clinical symptoms started earlier in *PSGL-1*$^{-/-}$ mice (Figure 7A and B). Examination of disease induced by 4% DSS showed that the disease progressed faster and was more severe in *PSGL-1*$^{-/-}$ mice: at day 5 of treatment KO mice had lost on average 20% of their initial weight (Figure 7A), had a high disease activity index (D.A.I.) (Figure 7B) and showed more severe colon inflammation than WT (Figure 7C).

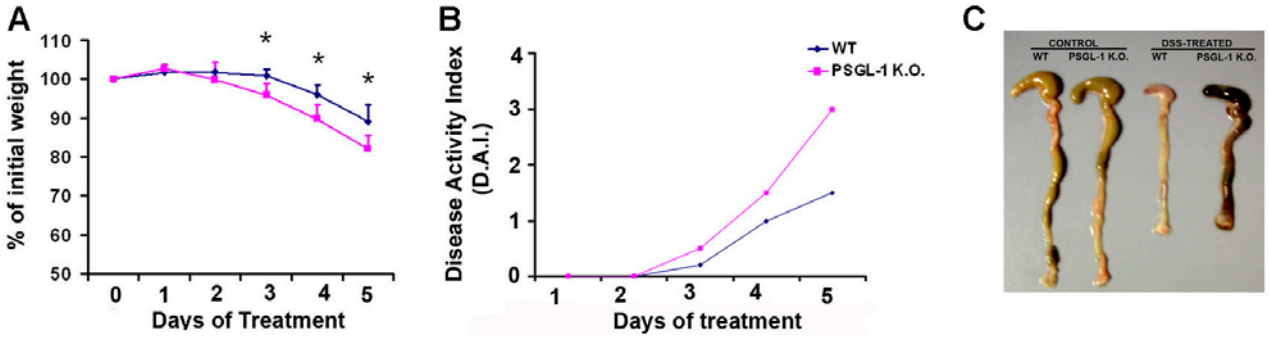

Figure 7. **Progression of acute colitis induced by DSS in *PSGL-1*$^{-/-}$ mice.** Acute colitis was induced in WT and PSGL-1 KO mice by including 4% DSS. A) Percentage of the initial weight from day 0 at day 5 of 4% DSS treatment. Data are means ± SD (n=5 mice) from a representative experiment of three; * $p<0.05$. B) Time course of disease activity index (DAI), scored by clinical symptoms (see Methods). C) Representatives images of colon obtained after 5 days of treatment with 4% DSS.

Moreover, three out of the 16 KO mice died on day 5 before the completion of analysis. Histological analysis showed that the colons of *PSGL-1*$^{-/-}$ mice were more distended and showed greater infiltration and a higher extent of crypt destruction than WT (Figure 8). These results indicate that *PSGL-1* deficient mice initiate colitis earlier and develop a more severe disease than WT mice.

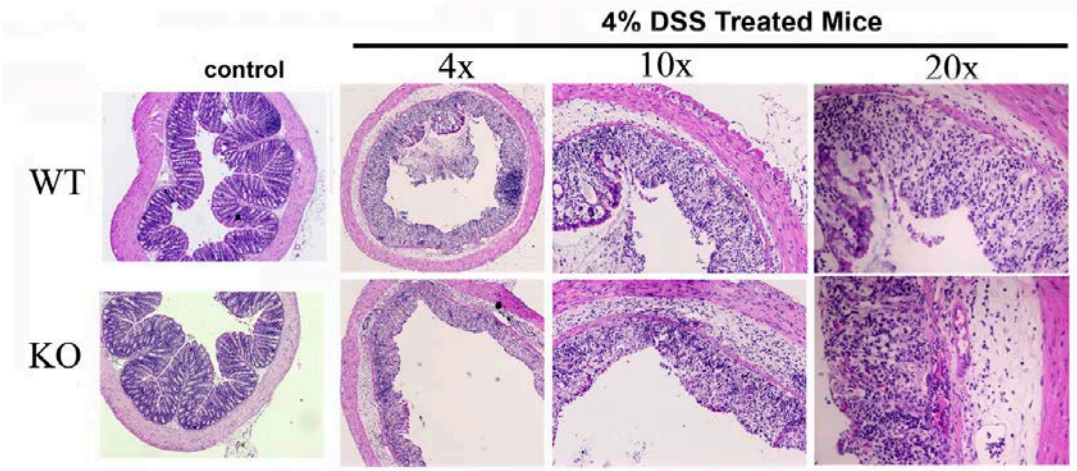

Figure 8. **Histologic analysis of disease severity.** Hematoxylin/Eosin staining of sections of colon obtained after 5 days treatment with 4% DSS.

4.1.5 Profile analysis of inflammatory immune cell populations in colonic LP affected by chemically induced colitis

We analyzed the colonic inflammatory cell infiltrate induced by DSS treatment. As shown in Figure 9, a diminished number of DCs and macrophages was observed in the inflammatory cell infiltrate of *PSGL-1*$^{-/-}$ mice.

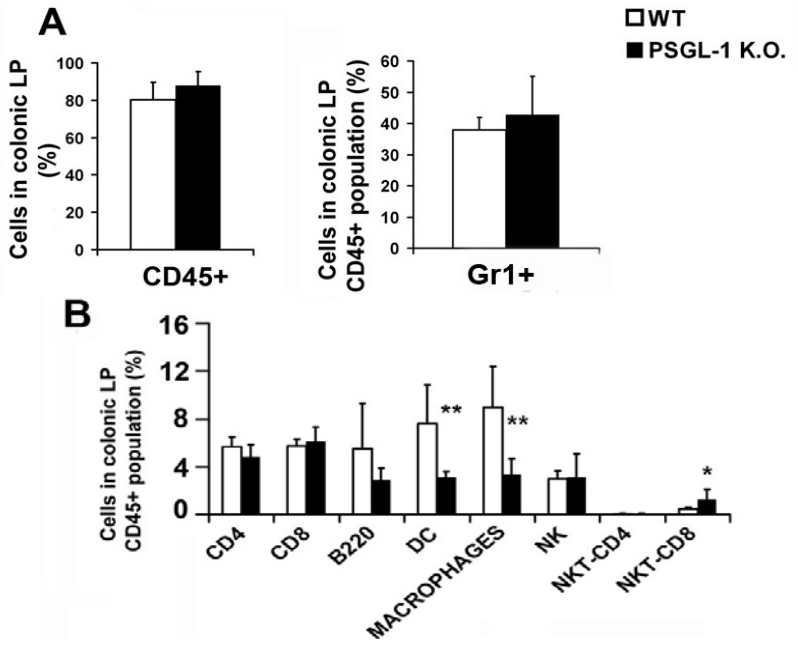

Figure 9. **Leukocyte infiltrate in the colonic LP of *PSGL-1*$^{-/-}$ mice after acute colitis induction.** Cell suspensions were obtained from colons of WT and PSGL-1 KO mice treated with 4% DSS (5 days). A) Percentage of CD45$^+$ cells in the LP infiltrate and the percentage of Gr1$^+$ cells in the CD45$^+$ population. B) Percentage of CD4$^+$ and CD8$^+$ T lymphocytes, B cells (B220$^+$), DC (CD11c$^+$), macrophages (F4/80$^+$) and NK cells (DX5$^+$) in the CD45$^+$ population.

Concerning the role of PSGL-1 exclusively on T lymphocytes and the development of UC, it has been reported that PSGL-1 deficiency exacerbates the development of inflammation in a mouse model of chronic ulcerative colitis (UC) induced by the adoptive transfer of naive *PSGL-1*$^{-/-}$ T cells into RAG-deficient hosts (Matsumoto et al, 2009). In fact, they observed that when PSGL-1 is absent, T cell enhances their adhesion and proliferative response. Moreover, other study suggested that PSGL-1 is not necessary to initiate ulcerative colitis and that the combination of other adhesion molecules play a more critical role for T cell trafficking and disease pathogenesis (Ostanin et al, 2007).

Immuno-blockade of PSGL-1 attenuated the established experimental colitis by reduction of leukocyte rolling and adhesion (Rijcken et al, 2004). In the same direction,

our results showed a reduction of leukocytes recruitment to the LP when 4RA10 blocking antibody was injected in WT mice; specifically macrophages, DCs and B cells. Accordingly, these populations were reduced in lamina propria under steady state and acute colitis induced with DSS in PSGL-1 knockout mice, but significant difference was observed neither in the cell number nor the percentage of CD4 and CD8 T cells. These results suggest that both models have differences regarding the conditional population; cell transfer model of colitis focus only in T cell capacity of proliferation while our DSS model reflects the effect of PSGL-1 absence in response to chemical injury of all the populations implicated in the establishment of the inflammatory response. However, the precise contribution of T cells and myeloid cells remains unknown.

Regarding the innate immune system, an enhanced proportion of $CD8^+$ NKT cells was detected in *PSGL-1*$^{-/-}$ upon DSS treatment (Figure 9). In addition, macrophages showed an increased expression of pro-inflammatory markers Gr1 and CD62L and enhanced expression of the activation marker CD69 (Figure 10).

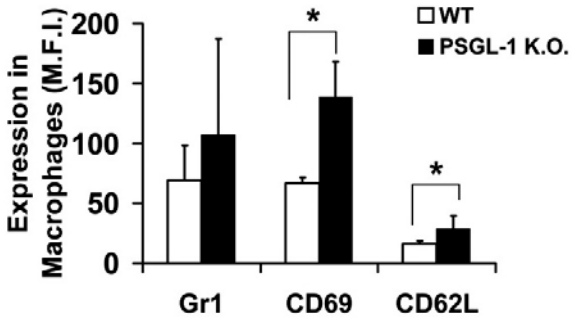

Figure 10. Expression of activation markers in macrophages after DSS treatment. Suspensions of F4/80+ cells were stained with antibodies for surface markers and analyzed by FACS (n=4)

In spite of the relatively low number of macrophages and DCs in the LP of PSGL-1 knockout mice under steady state conditions, these animals rapidly developed an aggressive form of colitis upon treatment with DSS, indicating that these mice have an enhanced ability to generate innate immune responses (Jurjus et al, 2004), what is in agreement with the activated phenotype of the LP macrophages.

The analysis of cytokine production showed that the percentages of macrophages producing IL-12, IL-4 and IFN-γ were higher in the *PSGL-1*$^{-/-}$ mice (Fig 11). In addition, the proportion of IL-10^+ DCs was lower in PSGL-1 deficient mice, whereas the percentage of IL-4-producing DCs was significantly enhanced (Figure 11).

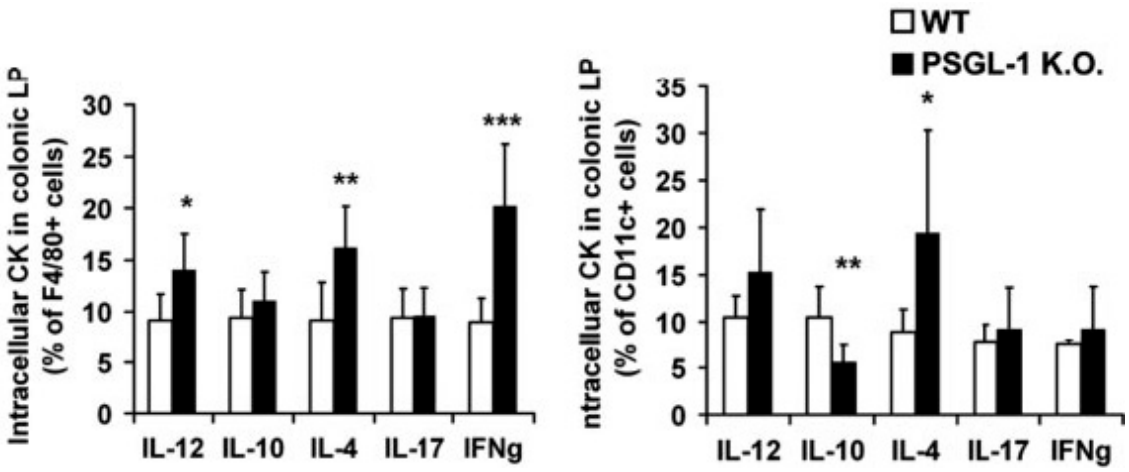

Figure 11. **Cytokine profile of innate immune cells in lamina propria in acute colitis.** Macrophage (left) and dendritic cells (right) subpopulations were analyzed by intracellular cytokine staining and FACs. In all cases, data are means ± SD from 6 mice

4.1.6 T cell responses in colonic LP during DSS-induced colitis

We next carried out a multi-cytokine analysis of colon homogenates. In this regard, it has been described that immunogenic DCs and activated macrophages are able to synthesize the pro-inflammatory cytokines IL-6 and IL-1, which induce the differentiation of Th17 cells (Sonderegger et al, 2008). Interestingly, IL-1α, IL-22 and IL-6 levels induced by DSS treatment were markedly higher in the colons of *PSGL-1*$^{-/-}$ mice (Figure 12).

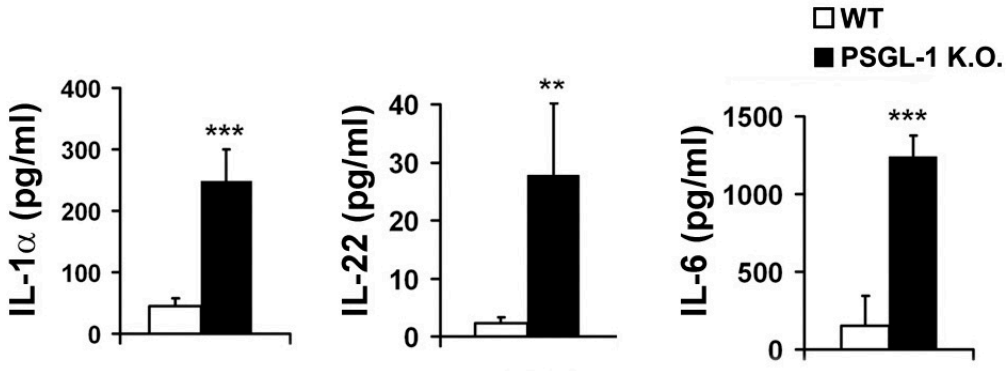

Figure 12. **Increased inflammatory cytokine levels in the colonic LP of PSGL-1-/- mice after acute colitis induction.** Colons were obtained from WT and PSGL-1 KO mice treated with 4% DSS. Total protein cell lysates were incubated with the antibody cocktail of the Th1/Th2/Th17 Flow Cytomix kit and analyzed by FACS. The panel shows the amounts of IL-1α, IL-22 and IL-6 found in 300 ng total protein (n=12 mice)

The analysis of T cell populations in LP of DSS treated mice showed an enhanced number of IFN-γ and IL-17 producing $CD4^+$ T cells (Th1, and Th17 cells, respectively) in PSGL-1 deficient mice (Figure 13 upper panel). Also a higher percentage of CD8+ T lymphocytes producing IFN-γ^+ and IL-17^+.

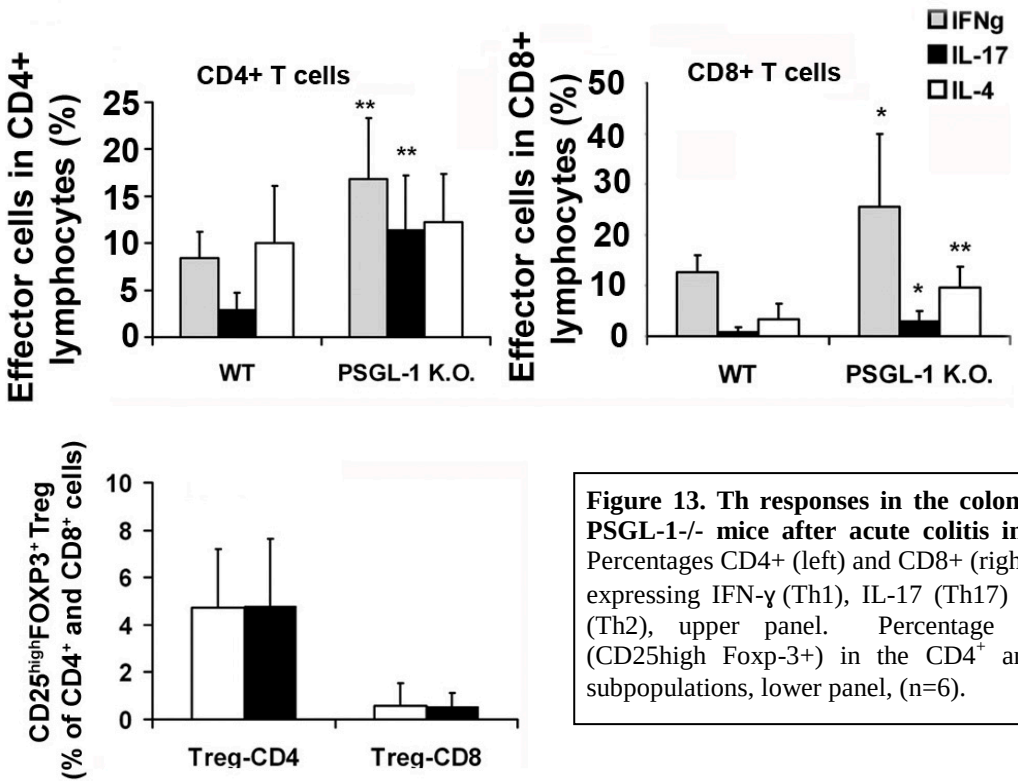

Figure 13. Th responses in the colonic LP of PSGL-1-/- mice after acute colitis induction. Percentages CD4+ (left) and CD8+ (right) T cells expressing IFN-ɣ (Th1), IL-17 (Th17) and IL-4 (Th2), upper panel. Percentage of Treg (CD25high Foxp-3+) in the $CD4^+$ and $CD8^+$ subpopulations, lower panel, (n=6).

Despite of an enhanced number of LP effector IFN-γ^+ and IL-17^+ lymphocytes in PSGL-$1^{-/-}$, these animals showed a similar proportion of Treg cells than WT mice (Figure 13 lower panel) which indicate an imbalance in the Treg/Th17 ratio. These data further support that PSGL-1 deficient mice have a defective generation of Treg cells (Urzainqui et al, 2007a), a phenomenon that very likely contributes to the enhanced tissue damage observed upon induction of acute colitis. Thus, it is very likely that in PSGL-1 deficient mice, the presence of increased proportion of activated macrophages under steady state conditions favors the differentiation of Th17 lymphocytes, in the presence of a triggering factor such as DSS. In turn, Th17 lymphocytes would be responsible for the observed over-production of IL-22, which along with IL-17 exert an important pro-inflammatory effect. Interestingly, recent studies in humans with IBD have detected increased levels of IL-17, IL-1 and IL-6 mRNAs in intestinal mucosa and a reduced ratio of circulating Treg/Th17 (Eastaff-Leung et al). In fact, these results are

in agreement with previous reports in the sense that T cells contribute to the increased disease of ulcerative colitis (Matsumoto et al, 2009). Here, in DSS acute colitis in PSGL-1 knockout mice, the basal enhanced activation of macrophages and DCs together with the reported higher proliferation rate of T cells and the insufficient generation of Tregs drives the inflammation in this model.

Altogether, our findings indicate that PSGL-1 engagement in DCs and macrophages is necessary for the regulation of innate immune cell responses as well as for the modulation of adaptive T cell responses and the initiation/resolution of inflammatory phenomena. We consider that this places PSGL-1 as a novel potential target for pharmacological intervention to ameliorate or resolve inflammatory diseases such as IBD.

4.2 HDAC6: Role at the exocytosis of lytic granules in CD8 T cells

4.2.1 Contribution of HDAC6 to the lytic protein repertoire.

In order to study the role of HDAC6 in the cytotoxic context we first performed a proteomic analysis of enriched microsomal fractions, associated to the centrosome, purified from wt and *hdac6*$^{-/-}$ CTLs by sucrose gradient ultracentrifugation. We found an altered composition in lytic granule-associated proteins in *hdac6*$^{-/-}$ cells. We detected a larger number of peptides for Perforin-1 and Granzymes A (isoform HF2), B, C and D; (Figure 14).

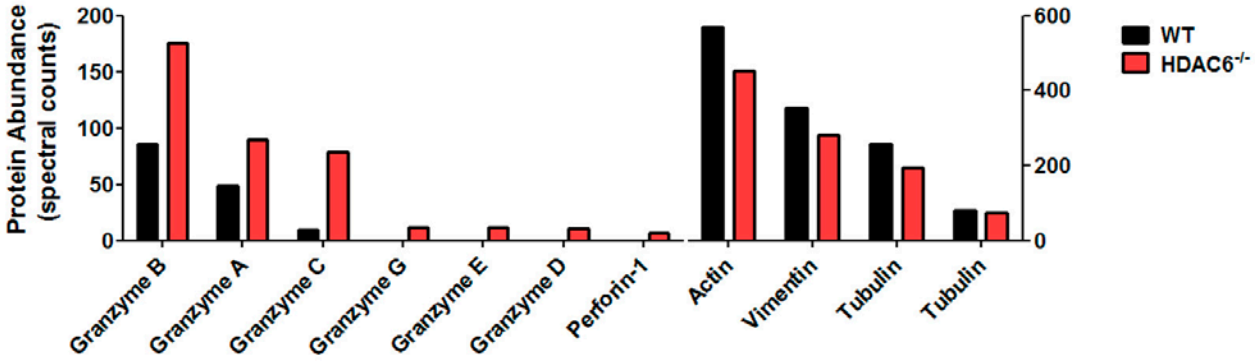

Figure 14. **Proteomic analysis from enriched microsomal fractions.** Enriched microsomal fractions purified by sequential subcellular fractionation and sucrose gradients from wild-type and *hdac6-/-* mature blast T cells previously activated *in vitro* and cultured with IL-2 during 12 days. Protein abundance (expressed in spectral counts) of a set of selected proteins is shown at the left; for comparison the abundances of other control proteins are also shown at the right.

Since these proteins are up-regulated by CD8 maturation into CTLs (Williams & Bevan, 2007), we analyzed the transcription levels of different proteins involved in the process (Figure 15). *granzyme B* showed an up-regulation, a slightly increment occurred in *perforin-1*, while *lamp1* and *cathepsin D* mRNAs showed no differences. *eomes* showed also an up-regulation, which is in accordance with its role in the regulation of *perforin* and *granzyme b* transcription.

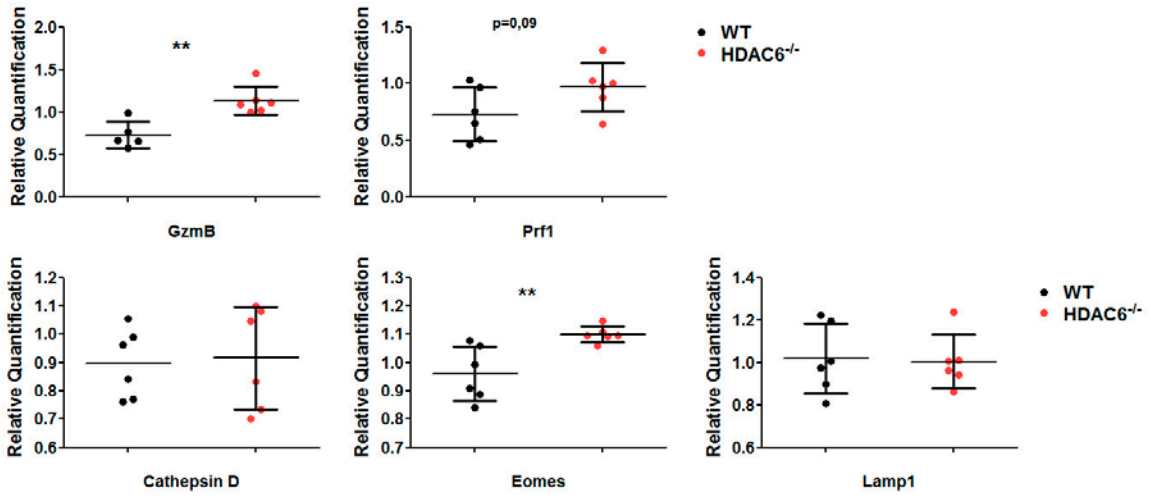

Figure 15. **Gene expression of cytotoxic genes.** The expression of these genes was assessed by qRT-PCR of RNA extracts from WT and *hdac6*$^{-/-}$ blast T cells, and normalized with *β-actin*, *β2 microglobulin* and *Ywhaz* expression (n=3 , from 2 independent experiments).

In fact, some HDACs have been implicated in the transcriptional regulation of these key proteins. HDAC1 deletion, e.g., increased Eomes expression in naïve CD8^{+} T cells at resting, while Prf1 seems to be up-regulated upon activation (Tschismarov et al, 2014).

4.2.2 HDAC6 deficiency reduces the cytolytic capacity of CD8^{+} T lymphocytes.

HDAC6 regulates the tubulin cytoskeleton through the post-translational control of α-tubulin acetylation (Valenzuela-Fernandez et al, 2008). To ascertain its role in CD8+ T cells, we examined the ability of cytotoxic T cells from *hdac6*$^{-/-}$ mice to kill target cells *in vitro*. Naïve CD8+ T cells from wild-type (WT) and *hdac6*$^{-/-}$ mice expressing the transgenic OVA-specific TCR receptor (OT-I) were stimulated with OVA257-264 (100 nM) and cultured for at least 7 days in presence of IL-2 to generate activated CTLs. Cell cytotoxicity was subsequently analyzed by survival of dye-labeled target cells pulsed or not with OVA257-264 peptide. *hdac6*$^{-/-}$ CTLs showed decreased killing activity at every target: CTL ratio tested (Figure 16A); this effect was also observed in CTLs from OT-I mice treated with the specific HDAC6 inhibitor tubastatin A, although to a lesser extent (Figure 16B).

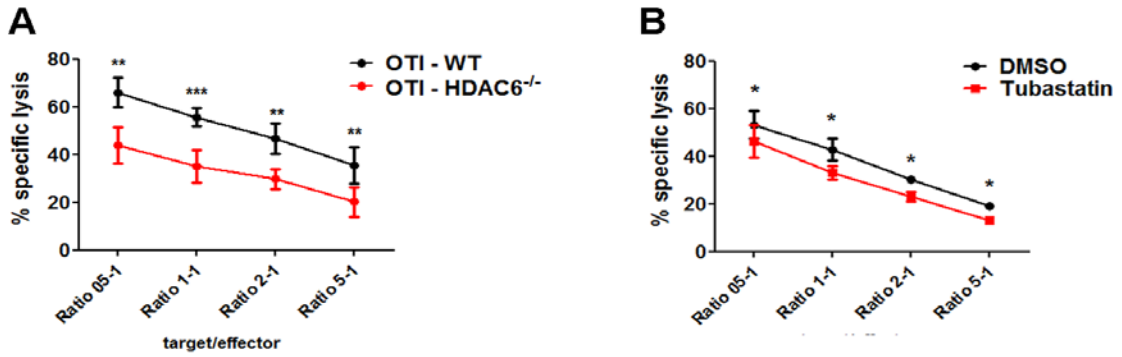

Figure 16. HDAC6 is required for cytotoxic activity. Cytotoxic assay showing specific lysis of EL4 target cells loaded with 500 nM OVA peptide (SIINFEKL) that were mixed with OT-I WT and *hdac6*$^{-/-}$ CTLs (A) (n=5); or CTLs treated with Tubastatin A vs vehicle (B) (n=3). Graphs show mean ± SEM of specific lysis for 5 h at the indicated target to effector ratios. All killing assay where performed in triplicate. *, P < 0.05; **, P < 0.01; ***, P < 0.001. Statistical analysis: unpaired T-test for OT-I WT and *hdac6*$^{-/-}$ and paired T-test for inhibitor treatment.

The decreased cytotoxicity observed does not correlate with the enhanced expression of Granzyme B, Perforin and Eomes found when HDAC6 is absent. These data point to a potential impairment in the transport and/or secretion process of lytic granules, instead of a defect in their biogenesis, since there seem to be enough lytic proteins in knockout cells to perform the cytotoxic function.

4.2.3 HDAC6 is required for degranulation in activated CTLs.

We next investigated the role of HDAC6 in lytic granule secretion by testing for the presence of CD107a at the CTL plasma membrane after TCR stimulation. Consistently with decreased cytotoxicity, *hdac6*$^{-/-}$ CTL showed a reduced expression of CD107a (lamp1) after 3h of degranulation using the same ratio of target and effector cells (Figure 17).

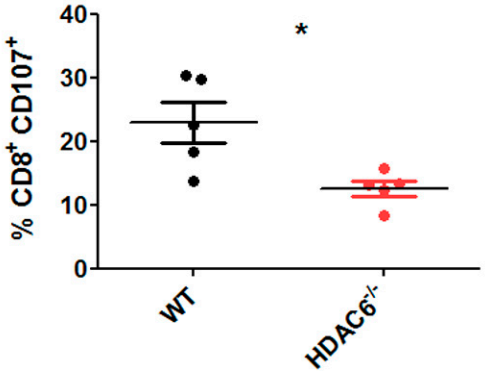

Figure 17. HDAC6 deletion decreases Lamp1 accumulation in membrane. Degranulation is shown as the percentage of CD107a^{+} (lamp1) cells detected by flow cytometry in activated vs non-stimulated WT and *hdac6*$^{-/-}$ CD8+ CTLs. *, P < 0.05. EL4 target cells loaded with 500 nM OVA peptide (SIINFEKL) that were mixed with OT-I WT or *hdac6*$^{-/-}$ CTLs during 3h in the presence of Monensin A (10µM) and CD107a^{+} Alexa 647 antibody.

Then, we assessed the proteins secreted from the lytic granules of $CD8^+$ T cells of control and *hdac6*$^{-/-}$ stimulated with with phorbol 12-myristate 13-acetate (PMA) for 1 h. We observed that Cathepsin D and Prf1 were decreased in supernatants from activated *hdac6*$^{-/-}$ CTLs (Figure 18).

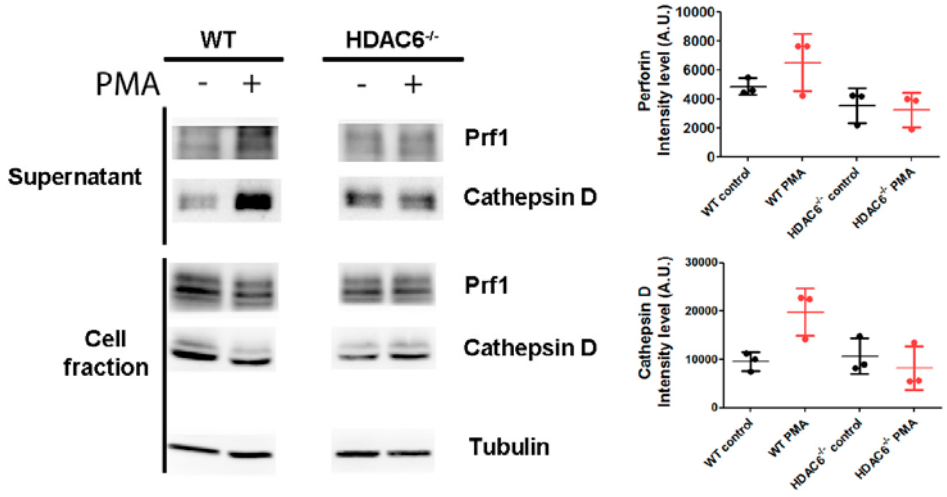

Figure 18. HDAC6 regulates the degranulation of CTLs. Exocytosis of lytic mediators is shown by western blot. Proteins released to the supernatant and collected in serum free medium are compared to the corresponding cell fraction upon PMA stimulation (1 h). A representative experiment is shown for mature $CD8^+$ CTLs from WT and *hdac6*$^{-/-}$. Tubulin is shown as loading control for endogenous protein level.

These results are in correspondence with lower lamp1 (CD107a) surface expression. Altogether our data demonstrate that HDAC6 inhibition by genetic or chemical means decrease the cytotoxic activity of CTLs, and suggest that HDAC6 may control exocytosis.

4.2.4 TCR signaling and IFN-γ production is not altered by HDAC6 absence in CTLs.

T-cell signaling induced by anti-CD3 plus anti-CD28, as determined by PLCγ1 (Figure 19A) and erk1/2 (Figure 19B) phosphorylation in $CD8^+$ T cells from *hdac6*$^{-/-}$ mice was comparable to control mice. These results suggest that the killing defect observed in HDAC6-deficient CTLs does not result from a general impairment of $CD8^+$ T cell function.

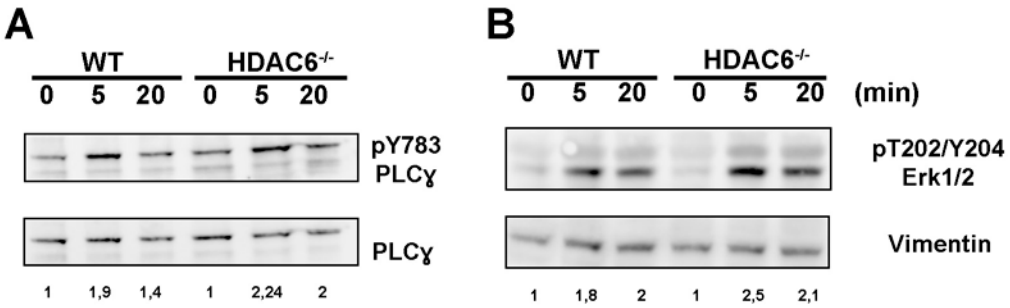

Figure 19. TCR signaling is conserved in *hdac6*$^{-/-}$ CTLs. Effector CD8^{+} CTLs isolated from WT and *hdac6*$^{-/-}$ mice were activated with anti-CD3/anti-CD28 antibodies for the indicated times. Cells were lysed and analyzed by western blot. Activation was analyzed by detecting PLCγ1 and erk1/2 phosphorylation at the indicated residues.

We next tested the role of HDAC6 in the expression of IFN-γ and found that the absence of HDAC6 did not affect secretion of IFN-γ and frequency of CTLs producing IFN-γ in response to polyclonal stimulation (Figure 20A and B). Likewise, treatment of CTLs from OT-I mice with tubastatin A had no significant effect on IFN-γ expression and secretion in response to OVA agonist peptide.

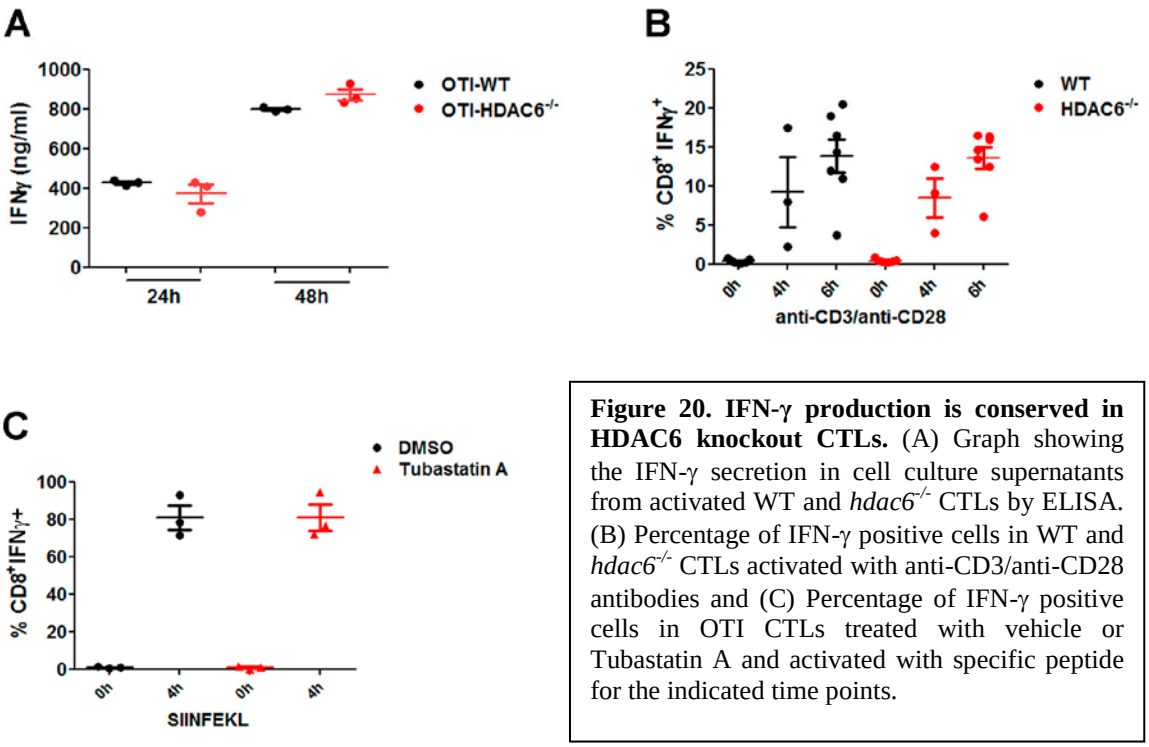

Figure 20. IFN-γ production is conserved in HDAC6 knockout CTLs. (A) Graph showing the IFN-γ secretion in cell culture supernatants from activated WT and *hdac6*$^{-/-}$ CTLs by ELISA. (B) Percentage of IFN-γ positive cells in WT and *hdac6*$^{-/-}$ CTLs activated with anti-CD3/anti-CD28 antibodies and (C) Percentage of IFN-γ positive cells in OTI CTLs treated with vehicle or Tubastatin A and activated with specific peptide for the indicated time points.

4.2.5 Defective *in vivo* and *ex vivo* killing in HDAC6 knockout mice

To assess the effector activity of *hdac6*$^{-/-}$ CD8^{+} T cells, we tested their ability to kill a specific target cell *in vivo* following immunization against specific antigens. WT and *hdac6*$^{-/-}$ mice were immunized with bone marrow-derived dendritic cells activated with LPS and pulsed with the OVA $_{257-264}$ peptide and *in vivo* killing activity against target cells pulsed or not with OVA peptide was analyzed seven days later.

Notably, *hdac6*$^{-/-}$ mice showed reduced specific killing of target cells *in vivo* (Figure 21A). However, the frequency of OVA-specific CD8^{+} T cells from the endogenous repertoire was not affected in the absence of HDAC6 (Figure 21B), suggesting that the cytotoxic function rather than the number of antigen-specific CTLs could underlie the defective killing activity in *hdac6*$^{-/-}$ mice.

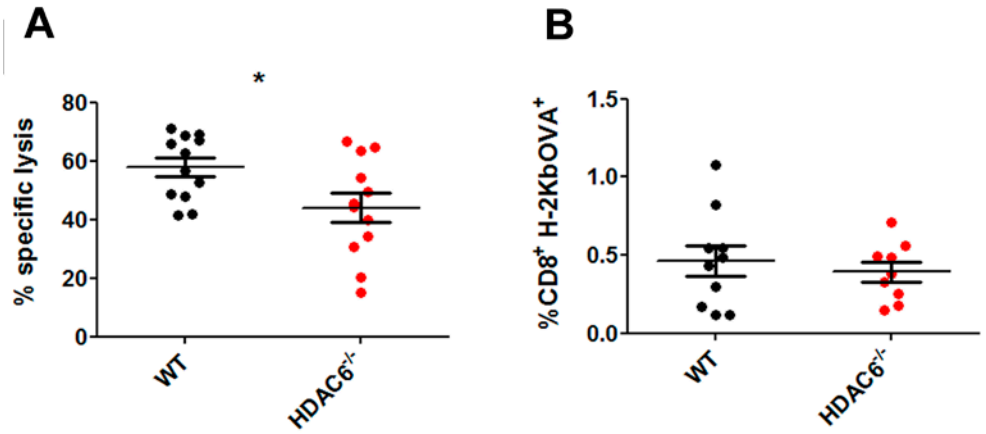

Figure 21. *In vivo* killing is impaired when silencing HDAC6. WT and *hdac6*$^{-/-}$ were immunized i.p. using bone marrow derived dendritic cell previously activated with LPS (1µg/ml) and loaded with the specific OVA$_{257-264}$ peptide in order to generate a specific CD8^{+} CTLs. 7 days after immunization, OVA pulsed target cells labeled with cell violet were injected ip, after 24h were recovered by intraperitoneal lavage and cell survival was assessed by FACs (A). Relative percentage of CD8^{+} H-2 Kb^{+} was determined as a control of avidity towards the OVA peptide (B), n=5, pool of 3 independent experiments. *, $P < 0.05$.

Next, we examined whether the decreased cytotoxic function of *hdac6*$^{-/-}$ CTLs resulted in an impaired ability to prevent morbidity/mortality during a viral infection. To restrict HDAC6 deficiency to CD8^{+} T cells, we adoptively transferred *rag1*$^{-/-}$ mice with WT or *hdac6*$^{-/-}$ CD8^{+} T cells and subsequently challenged with a fully replicative vaccinia virus (VACV) WR strain. This infection model mimics the immunological and clinical features of smallpox vaccination in humans (Mota et al, 2011). The *rag1*$^{-/-}$ mice passively-transferred with *hdac6*$^{-/-}$ CD8^{+} T cells showed increased morbidity at days 9

and 11 post-infection (p.i.) compared to *rag1*$^{-/-}$ mice transferred with WT CD8^{+} T cells (Figure 22A).

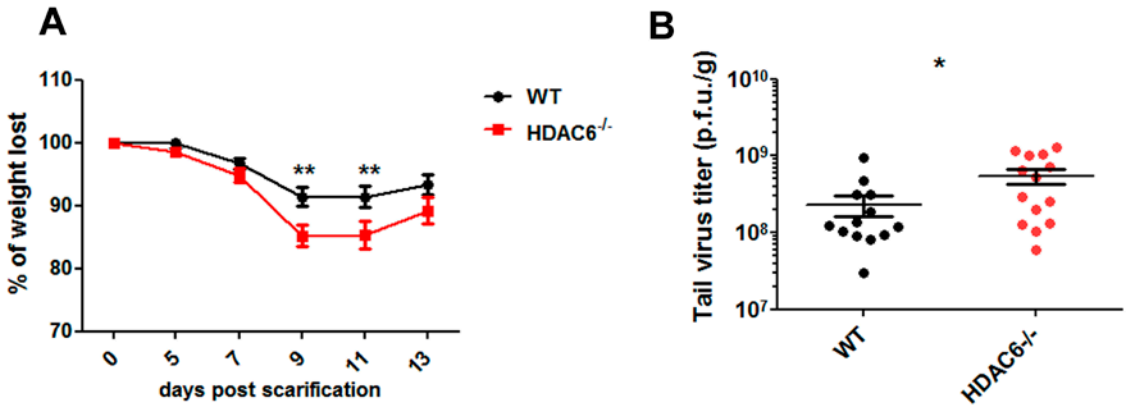

Figure 22. Vaccinia infection disease progression. (A) *rag1*$^{-/-}$ mice were inoculated intravenously with 0.8x10^{6} CD8+ naïve cells; all animals were infected with VACV-WR by tail scarification and weighted every 2 days. Graphs represent means. Bars, SEM. **, P < 0.01. (B) Titration of viral particles from scarified tails from WT and hdac6-/- mice upon 13 d.p.i. Tissue from tails was disaggregated. CV-1 cells were infected in vitro with different dilutions of tissue extracts, fixed and stained. Colonies of VACV-infected cells were counted.

Virus titration from the lesional tissue demonstrated that *hdac6*$^{-/-}$ immune cells exerted less efficient virus clearance (Figure 22B). The model of infection described by (Mota et al, 2011) in *rag1*$^{-/-}$ mice without cell transfer produced a severe disease, with uncontrolled viral replication at the inoculation site and dissemination to internal organs. Eventually, these mice reached 100% mortality before the day 20 post infection. *rag1*$^{-/-}$ mice are T and B cell deficient, due to a disruption of the recombination activation gene 1, responsible for somatic recombination that occurred in T and B cells precursors, but they retain normal levels of population such as macrophages, dendritic and natural killer cells. The development of generalized disease implicated disseminated poxvirus lesion in the face, forepaw and tail, which culminate in mortality. This confirms the essential role of adaptive immune population in host to control the replication of VACV in the murine model of scarification.

Consistent with our findings on the lack of effect in endogenous antigen-specific CD8^{+} T cell numbers, CTL expansion tracked at day 13 p.i. was not affected in the absence of HDAC6 (Figure 23, left). The proportion of these cells at early (5 d.p.i) and late stages (13-30 d.p.i.) of the disease were similar for WT and *hdac6*$^{-/-}$ mice (tested in peripheral blood or spleen, respectively; Figure 23, right).

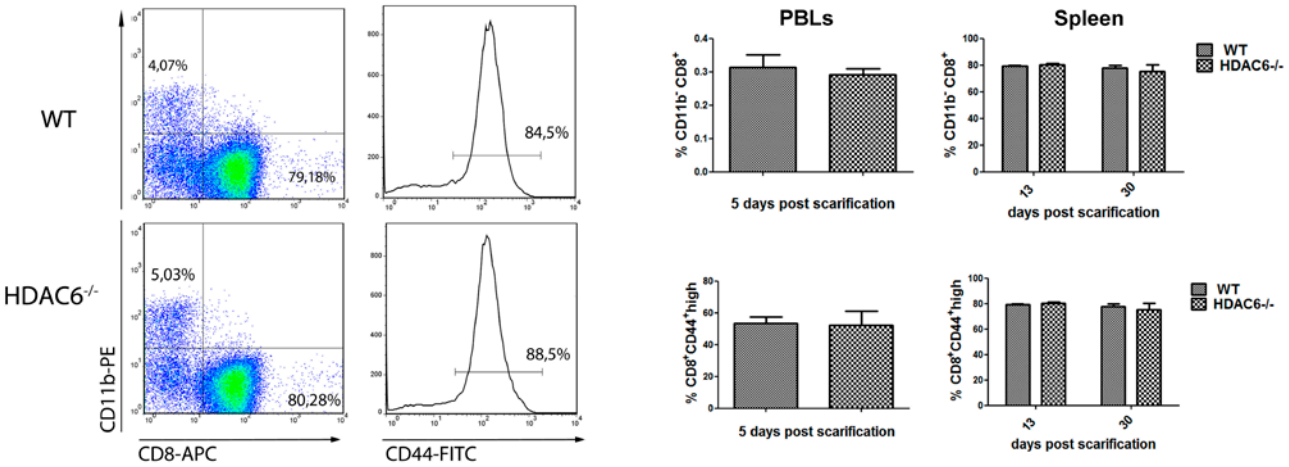

Figure 23. **CD8$^+$ T cell *in vivo* proliferation.** Percentage of CD8$^+$CD44$^+$ isolated from blood (5 d.p.i.) and spleen (13 and 30 d.p.i.) of Rag1-/- mice and analyzed by FACs. CD11b was used as negative control, n=5.

Independently, we tested the *in vivo hdac6*$^{-/-}$ CD8$^+$ T cell proliferation using a OTI- OVA system. WT mice were injected in the foothpath with a WW-VACV (vaccinia virus specific for a OVA peptide); then labeled naïve CD8$^+$ T cell from WT and *hdac6*$^{-/-}$ OT-I mice were transferred i.v. The proliferation rate was comparable, or even increased (division 4) in *hdac6*$^{-/-}$ compared to WT (Figure 24).

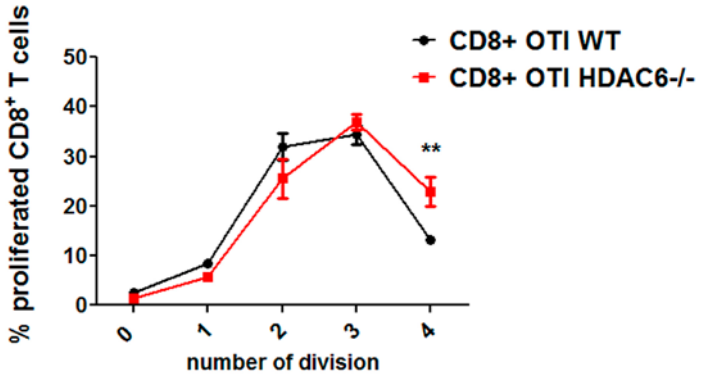

Figure 24. **CD8$^+$ T cell *in vivo* proliferation.** WT and HDAC6$^{-/-}$ CD8 naïve T cell were labeled with Cell violet and injected i.v. in CD45.2 receptors, immediately mice were scarified. Expanded CD8 T cells were recovered at day 4, n=5. * P < 0.05.

By contrast, *in vivo* assays using *hdac6*$^{-/-}$ CD8^{+} T cells in response to a different viral infection model like LCMV (lymphocytes choriomeningitis virus strain) infection showed similar results of *hdac6*$^{-/-}$ CD8^{+} T cell respect to WT (data not show). Our hypothesis is that this phenotype is due, in part, to a compensatory effect probably coming from other histone deacetylase and to the specific activation of other members of the family . In fact, reports studying HDAC1 showed a deficient differentiation of effector CD8^{+} T cell and a reduced anti-viral response upon LCMV infection and impaired expansion. *In vitro*, CTLs were largely unaffected and this has been attributed to a compensatory effect from another member family of deacetylase like HDAC2 which partially compensates for loss of HDAC1 and thus mask potential functional consequences of HDAC1 deletion (Tschismarov et al, 2014). Thus, it is likely that this HDAC I family member partially compensates for loss of HDAC6 and thus masks potential functional consequences of HDAC6 deletion. These *in vivo* results emphasize the role of HDAC6 in the CD8^{+} T cell-dependent protection against *Vaccinia virus* infection. The differentiation of effector CD8^{+} CTLs seems to be unaffected by HDAC6 deficiency, since the proliferation rate and cell proportions are similar to wild type.

4.2.6 Impairment at the late stage of lytic granule exocytosis

CTL killing is limited to target cells (and not neighbor cells) by the confinement of secretion to the immune synapse that is established between the CTL and the target cell (de Saint Basile et al, 2010). Interestingly, perforin and lamp1 (CD107a) intracellular co-localization was affected in *hdac6*$^{-/-}$ CTLs conjugated with target cells, pointing to a de-regulated localization of perforin in these cells (Figure 25; images and lower graphs). This corresponds with the decreased secretion of lytic proteins in response to PMA in the *hdac6*$^{-/-}$ CTLs and suggests that HDAC6 regulates exocytosis of lytic granules (Figure 18).

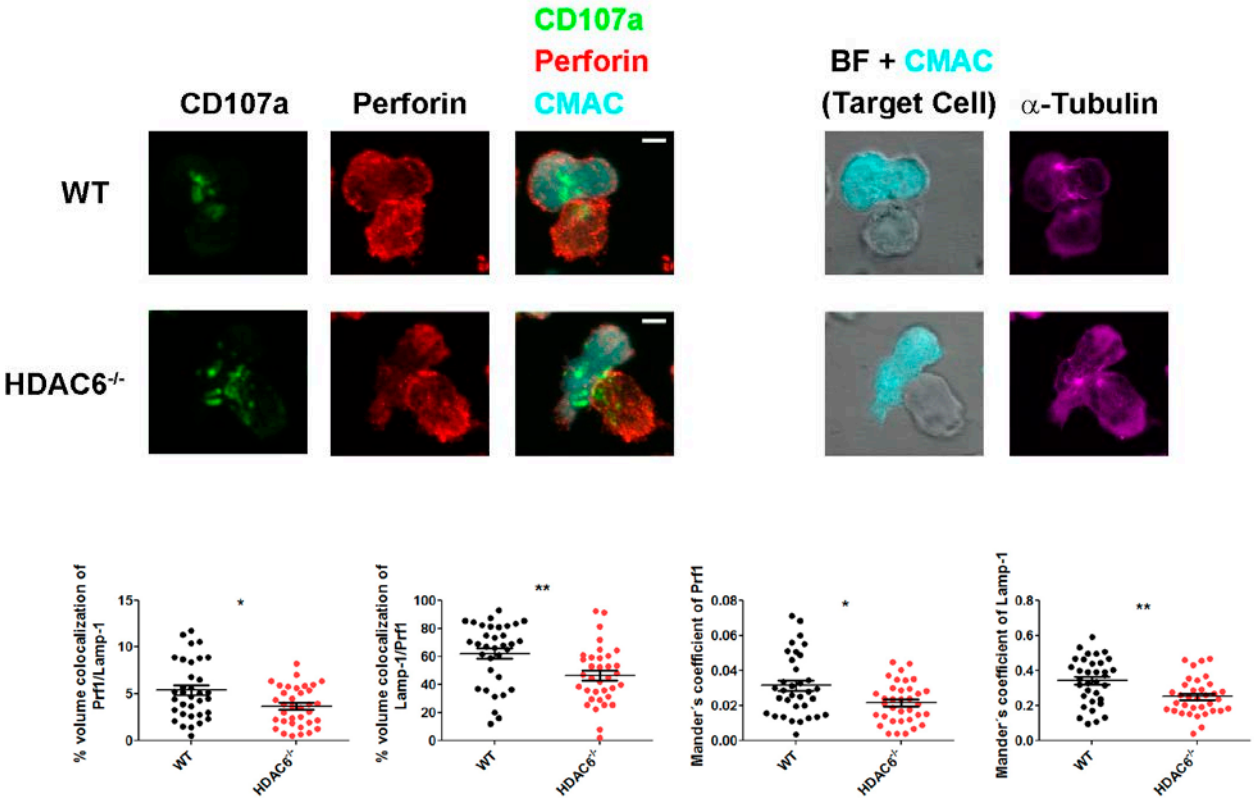

Figure 25. **CD107a and Perforin co-localization upon conjugate formation.** Images from confocal microscopy of CTLs derived from WT and hdac6-/- OT-I mice and conjugated with CMAC cell tracker-loaded, OVA pre-pulsed target cell EL4 (500 nM SIINFEKL, Cyan). Conjugate formation was allowed for 15 min and cells were fixed, permeabilized and stained with anti-CD107a, anti-perforin (green and red, respectively; left panel) and α-tubulin (magenta; right panel) antibodies. Fluorescence images correspond to maximal projections of equal number of slices from confocal Z-stacks. BF images correspond to a unique plane. Scale bar, 4 µm. Lower graphs show the co-localization of Lamp1 (CD107a) and Perforin1 in cells (n= 35 cells for WT and hdac6-/- CTLs). *, P < 0.05; **, P<0.01; Mann-Whitney test.

4.2.7 Efficient MTOC translocation in hdac6-/- CTLs

Defects in lytic granule polarization to the contact are with the target cell could be explained by a impaired MTOC translocation. However, the efficient translocation of centrosome to the contact area with the target cell observed in *hdac6*$^{-/-}$ CTLs (Figure 26) suggests that the defective exocytosis may rely on the movement of granules themselves.

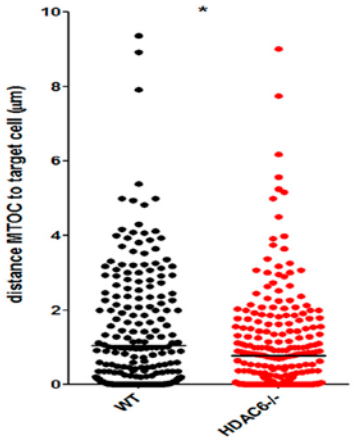

Figure 26. MTOC distance to APC. Quantification of MTOC translocation, shortest distance of MTOC to the APC surface in the contact area (n>270 from 3 independent experiments); *, P < 0.05. Unpaired T-test.

This is in accord with previous studies described the lack of effect on MTOC polarization upon immune synapse in $CD4^+$ T cells when were treated with TSA, while the HDAC6 over-expression prevented its polarization (Serrador et al, 2004).

4.2.8 Critical role of HDAC6 in the lytic granule release.

We thus monitored the dynamics of granule release by Total Internal Reflection Fluorescence microscopy (TIRFm). Cells were loaded with a pH-dependent, lysosomal tracker that allows the visualization of the lysosomal-related lytic granules. The CTLs were allowed to settle on an anti-CD3 + anti-CD28-coated surface. CTLs form an IS-like structure in which the dynamics of lytic granules can be tracked at the intracellular proximal region beneath the plasma membrane (Figure 27).

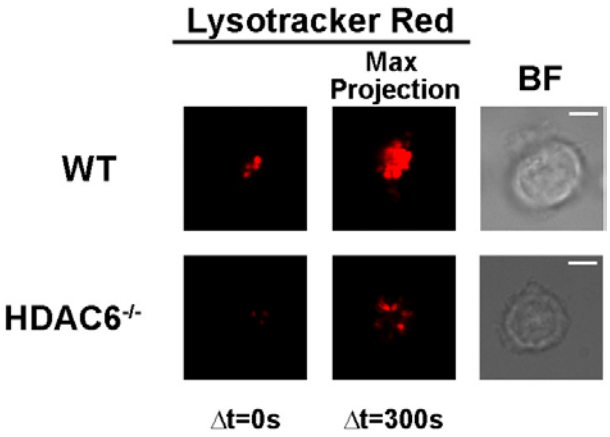

Figure 27. Representative TIRFm images of Lysotracker red-loaded CTLs. $CD8^+$ CTLs from WT and *hdac6*$^{-/-}$ mice were allowed to settle on anti-CD3/anti-CD28-coated glass-bottom chambers maintained at 37ºC and 5% CO2. Video recording was initiated upon cell adhesion with the stimulating surface. Images were acquired for 5 min every 0.5 s at 90 nm of penetrance with 561 nm laser excitation. Scale bar, 4 µm

These experiments revealed significant changes in the distribution of the lytic granules and their dynamics, with a marked decrease in the number of lytic granules detected at the IS-like in *hdac6*$^{-/-}$ suggesting alterations to the active transport of the granules from the centrosomal region to the plasma membrane. Indeed, the mean fluorescence intensity detected for *hdac6*$^{-/-}$ granules was lowered, which suggests a higher pH and therefore a different degree of maturation, although the lytic granules

displayed similar sizes in WT and *hdac6*$^{-/-}$ (Figure 28A).

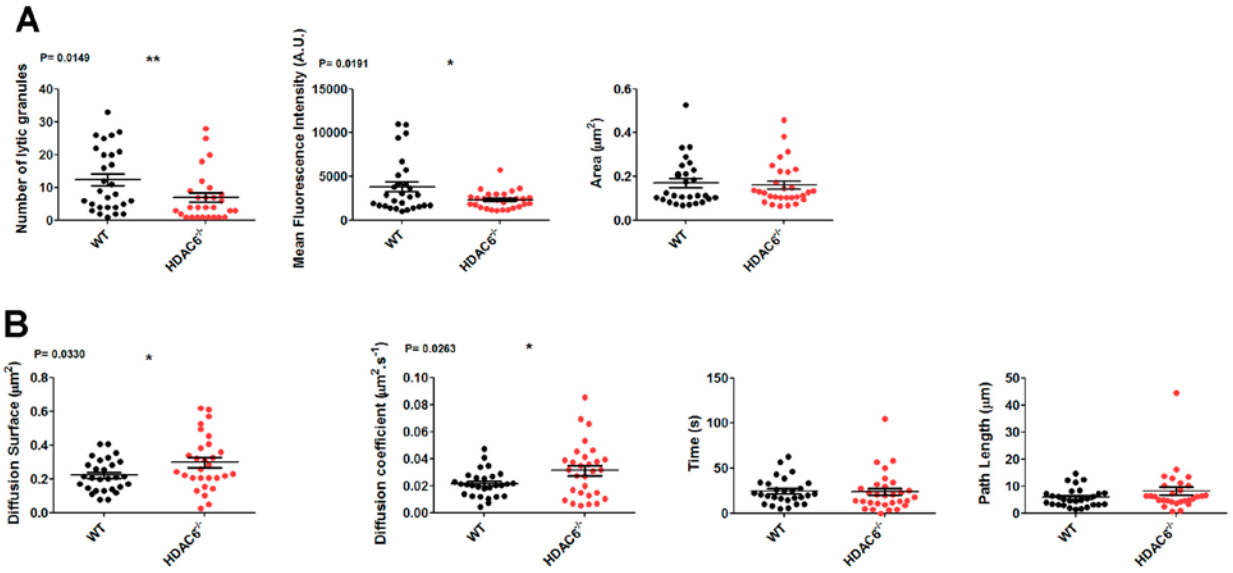

Figure 28. **Dynamics quantification of lytic granules.** The number of lytic granules, area of particles (size), path length, duration (time) (A) and diffusion surface, diffusion coefficient and mean fluorescence intensity (B), at the IS-like was quantified for each cell. n=28 cells of each genotype were analyzed for the entire time-lapse from 3 independent experiments.

The most remarkable difference pertained to the xy distribution of the lytic granules, which was wider (diffusion surface) in the *hdac6*$^{-/-}$ CTLs, therefore showing enlarged diffusion coefficient maintaining similar duration times and paths length (Figure 28B). These data suggest that the lytic granules from *hdac6*$^{-/-}$ CTLs are not properly targeted to the contact with the target cell and dock deficiently at the plasma membrane.

4.2.9 HDAC6 binds kinesin-1 motor and links dynactin complex subunits

Tubulin motors control the delivery of lytic granules to the plasma membrane. Whereas dynein controls granule targeting to the centrosome (Burkhardt et al, 1993), kinesin-1 together with Slp3/Rab27a directs terminal transport to the plasma membrane for exocytosis (Kurowska et al, 2012). In this report Slp3 acts as an effector of Rab27a during the terminal process of exocytosis in cytotoxic T lymphocytes, and forms a complex with kinesin-1 through binding to the tetratricopeptide repeat of the light chain subunit (KLC1). This complex is a determinant step for polarized lytic granules to reach de membrane (docking) and eventually release their content at the immune synapse. In fact, it has been previously proposed that for the terminal step of lytic granules exocytosis, they must undergo a maturation step via the fusion of lytic granules with an

endosomal compartment bearing Rab27a, Munc13-4, and Slp exocytic effectors (Menager et al, 2007). This occurs once both type of organelles have been brought close to the immune synapse, which support the idea of Rab27a-Slp3–kinesin-1 complex acting in the terminal transport once lytic granules have acquired the exocytic machinery.

Dynactin may also be part of this complex, linking the cargo to kinesin-1 motor, thereby powering vesicles towards the plus end of microtubules (Haghnia et al, 2007; Hendricks et al, 2010). There is a debate of whether α-tubulin Lys-40 acetylation directly mediated the transport and velocity of Kinesin-1 to the microtubule track. First, it has been reported that the loss of α-tubulin acetylation at Lys-40 influences in the biding and motility of kinesin-1 in cultured neuronal cells (Reed et al, 2006). Second, by comparing the kinesin velocity and affinity to the track on microtubules reconstituted from acetylated and deacetylated porcine tubulin, it was observed that tubulin acetylation alone does not affect kinesin-1 motility (Walter et al, 2012).

We hypothesized then, that HDAC6 may regulate the movement and delivery of the lytic granules at the IS through a direct kinesin-1 interaction. Using a biochemical approach, we observed that HDAC6 formed a complex with kinesin-1 light chain (KLC1) when cells were triggered with anti-CD3 + anti-CD28 (Figure 29A).

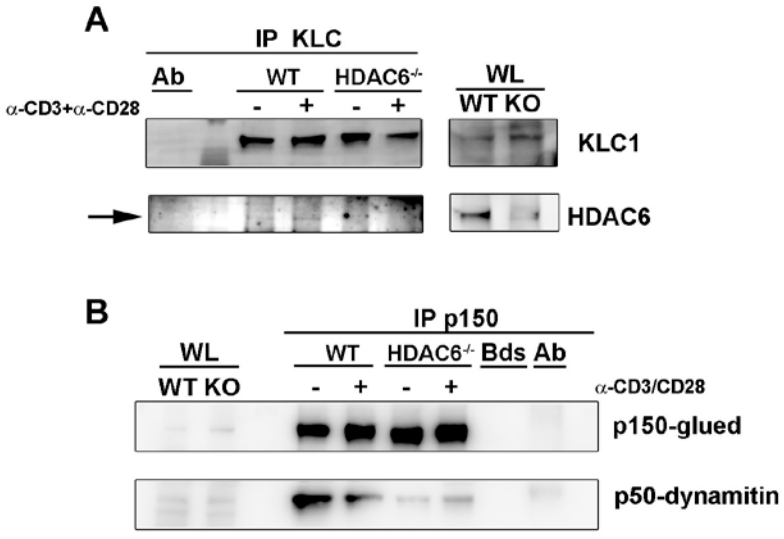

Figure 29. **Western blots showing co-immunoprecipitation of transport complexes.** Cell lysates of resting or anti-CD3+anti-CD28-activated WT and *hdac6*$^{-/-}$ mature CD8+ CTLs (15 min). Antibodies anti-kinesin light chain 1 subunit from kinesin complex (KLC1) or anti-p150 glued subunit from dynactin complex (p150) were used. Immunoprecipitates were processed and blotted against anti-KLC1 and HDAC6 (A) or p150 and p50 dynamitin subunits from dynactin complex (B) antibodies.

Moreover, interaction of the kinesin-activator complex dynactin subunits p150-glued and p50-dynamitin was impaired in the absence of HDAC6 in T cells (Figure 29B).

It has been described, in NK cells, that myosin IIA facilitates lytic granule transit through the F-actin-rich area in the surrounding of the immune synapse (Andzelm et al, 2007). This evidence together with the fact that the strength of TCR dictates the path the lytic granules take to position within the immune synapse suggest that, depending on the signaling strength, kinesin-1 and myosin IIA play distinct role in directing lytic granules transport to secretion sites. The possibility that Kinesin-1 and/or its association with dynactin could be tuned by HDAC6 underscores this interaction as a possible regulator of the recently described kinesin-1/Slp3/Rab27a complex that mediates transit step to membrane.

In summary, our data support a specific role for HDAC6 in the intracellular localization of lytic mediators and, particularly, in the process of their secretion. Therefore, the catalytic and scaffold activities of HDAC6 may act at multiple levels in the control of cytotoxic-related pathways and make HDAC6 a potential candidate to target CTLs in specific diseases, as a therapeutic strategy.

CONCLUSIONS

5. CONCLUSIONS

1 *PSGL1*$^{-/-}$ mice show an altered distribution of leukocyte subpopulations in the lamina propria at steady state, with decreased proportion of innate immune cells (DCs, macrophages and NKs).

2 The cytokine profile and pro-inflammatory phenotype of myeloid and T cells support a role for PSGL-1 on the regulation of the balance between tolerance and immunity.

3 PSGL-1 tolerogenic role in DCs and macrophages is confirmed *in vivo* by administration of 4RA10 antibody that reduces the MHC-II expression in these populations, acting as an agonist of P-selectin.

4 *PSGL-1*$^{-/-}$ mice develop an earlier and exacerbated colitis induced by DSS treatment in accordance with the unbalance proportion of Treg/T effector cells at the previous steady state.

5 HDAC6 deficit alters the gene expression of cytotoxic proteins and impairs the cytolytic capacity of CD8^{+} T lymphocytes by affecting the secretion of lytic granules.

6 Activation of CD8^{+} T cells and IFN-γ secretion the TCR in the absence of HDAC6 were comparable to WT.

7 CD8^{+} *HDAC6*$^{-/-}$ T cells have a reduced ability to prevent morbidity/mortality during viral infection in *Rag1*$^{-/-}$ mice when infected with vaccinia virus.

8 Granule number, intensity and spreading at the proximal region to the plasma membrane are altered in CTLs in the absence of HDAC6.

9 HDAC6 interacts with kinesin-1 light chain and dynactin complex to properly transport lytic granules to their terminal secretion domain in order to complete exocytosis.

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APPENDIX

7. Appendix – List of publications

This work resulted in the following publications:

- P-selectin glycoprotein ligand-1 modulates immune inflammatory responses in the enteric lamina propria. Nuñez-Andrade N, Lamana A, Sancho D, Gisbert JP, Gonzalez-Amaro R, Sanchez-Madrid F, Urzainqui A. J Pathol. 2011 Jun;224(2):212-21. doi: 10.1002/path.2850. Epub 2011 Mar 22.

- Development of an autoimmune syndrome affecting the skin and internal organs in P-selectin glycoprotein ligand 1 leukocyte receptor-deficient mice. Pérez-Frías A, González-Tajuelo R, Núñez-Andrade N, Tejedor R, García-Blanco MJ, Vicente-Rabaneda E, Castañeda S, Gamallo C, Silván J, Esteban-Villafruela A, Cubero-Rueda L, García-García C, Muñoz-Calleja C, García-Diez A, Urzainqui A. Arthritis Rheumatol. 2014 Nov;66(11):3178-89. doi: 10.1002/art.38808.

- HDAC6 regulates the dynamics of lytic granules in cytotoxic T lymphocytes. Norman Núñez-Andrade, Salvador Iborra, Antonio Trullo, Olga Moreno-Gonzalo, Enrique Calvo, Elena Catalán, Gaël Menasche, David Sancho, Jesús Vázquez, Tso-Pang Yao, Noa Beatriz Martín-Cófreces, Francisco Sánchez-Madrid. (*Under review*).

Other publications:

- Mitochondrial Respiration Controls Lysosomal Function during Inflammatory T Cell Responses. Baixauli F, Acín-Pérez R, Villarroya-Beltrí C, Mazzeo C, Nuñez-Andrade N, Gabandé-Rodriguez E, Ledesma MD, Blázquez A, Martin MA, Falcón-Pérez JM, Redondo JM, Enríquez JA, Mittelbrunn M. Cell Metab. 2015 Sep 1;22(3):485-98. doi: 10.1016/j.cmet.2015.07.020. Epub 2015 Aug 20.